VACCINES CONTAINING RECOMBINANT PILIN AGAINST *NEISSERIA GONORRHOEAE* OR *NEISSERIA MENINGITIDIS*

Field of the Invention

This invention relates to the use of recombinant pilin proteins in vaccines to protect against disease caused by *Neisseria gonorrhoeae* or *Neisseria meningitidis*.

Background of the Invention

Neisseria gonorrhoeae (*N. gonorrhoeae*) and *Neisseria meningitidis* (*N. meningitidis*) are Gram-negative cocci. *N. gonorrhoeae* and *N. meningitidis* are genetically very closely related, but the clinical manifestations of the diseases they produce are very different. *N. gonorrhoeae* causes gonorrhea, while *N. meningitidis* causes meningococcal meningitis. These bacteria of the genus *Neisseria* inhabit mucosal surfaces of the body.

Type IV pili are nonflagellar hairlike structures on the surface of numerous Gram-negative bacteria, including *Dichelobacter* (formerly *Bacteroides*) *nodous, Eikenella corrodens, Kingella denitrificans, Moraxella bovis, M. lacunata, M. nonliquefaciens, N. gonorrhoeae, N. meningitidis,* and *Pseudomonas aeruginosa* (Bibliography Entries 1,2). The toxin co-regulated pili from *Vibrio cholerae* and the bundle forming pili of enteropathogenic *Escherchia coli* exhibit a limited number of similarities to type IV pili and are considered to be more distantly related (1,2). For both *N. gonorrhoeae* and *N. meningitidis*, piliated bacteria adhere to a variety of epithelial cells of human origin much more avidly than do

nonpiliated cells, and thus the pili are thought to act as virulence factors by anchoring the organisms to mucosal infection sites.

Type IV pili are 5-7 nM in width and up to 5 μM in length. The pilin protein subunits are linked in tandem to form long, thin polymers. In the case of the pathogenic *Neisseria*, the pili are apparently homopolymeric in nature, being comprised of a single structural subunit, the pilin protein. The pilin protein has a molecular weight of 13,000 to 22,000 daltons (1,2,3).

In nature, the pilin protein is assembled into a helical structure called a pilus (plural: pili) at the bacterial outer membrane that has a molecular weight of approximately 10^7 daltons. When purified pili are dialyzed against pH 12 phosphate buffer, the intact pili are irreversibly dissociated into aggregates of the pilin protein which are called pilin oligomers (4). These pilin oligomer aggregates (molecular weight of approximately 600,000 daltons) are much smaller in size than the intact pilus.

N. gonorrhoeae expresses a single pilin protein which was first isolated and sequenced by Schoolnik and co-workers (5). The gonococcal pilin protein consists of three regions: (a) the highly conserved amino terminal region (residues 1-53); (b) the middle third (residues 54-124) which exhibits a limited amount of sequence variation and (c) the carboxy third of the protein (residues 125-160) which contains a highly variable disulfide loop.

During the course of natural infections, pili undergo high frequency phase and antigenic variation (3,6). The genetics of this variation are extraordinarily complex and have been extensively studied. Each strain of *Neisseria gonorrhoeae* has the

ability to change the primary amino acid sequence of the pilin molecule, and thus the antigenic nature of its pili. The molecular mechanism responsible for this variation involves a nonreciprocal recombination event between the expression locus (*pilE*) and numerous (17 to 19), promoterless, silent (*pilS*) genes (6). Pilin sequences (or portions thereof) move from the *pilS* loci into an expression locus to generate new pilin variants, which in turn enables gonococci to express an extremely large number of pilin proteins.

In contrast to *N. gonorrhoeae*, *N. meningitidis* expresses two distinct classes of pilin named class I and class II. As in the gonococci, the meningococcal class I pili undergo antigenic and phase variation (3). Class I pilins have been shown to be similar to gonococcal pilin in terms of molecular weight (17-20 kd) and reactivity with a monoclonal antibody (SM1) which binds to a highly conserved epitope on the gonococcal pilin (3). A number of class I pilins have been cloned and the amino acid sequences have been shown to have a high degree of similarity to the sequence of the gonococcal pilin (7). In contrast, class II pilins do not react with the SM1 antibody and have a lower molecular weight (13-16 kd). Several strains expressing class II pilin have been shown to react with a polyclonal antisera directed against gonococcal pili (3). Aho et al. (7) have recently determined the sequence of a class II pilin. The first third of the neisserial pilins are essentially identical. The class II pilin protein differs from the class I pilin and gonococcal pilin proteins in the hypervariable region of the protein where a large deletion has occurred. Achtman et al. (8), using monoclonal antibodies specific for class I or class II pilins, demonstrated that some serogroup A

isolates from Africa bound both antibodies. It has been demonstrated that strains expressing class II pili also have truncated, silent class I pilin genes (7). Taken together, these data suggest the possibility that a single meningococcal cell might express both pilin proteins simultaneously.

Because pili are believed to mediate the initial contact with mucosal cells, there has been considerable interest in using these structures as vaccine antigens to prevent disease caused by piliated bacteria. Pili vaccines against traveler's diarrhea and gonorrhea have been tested in human beings (9). However, to date, they have been efficacious only against homologous strains. A number of pili-based vaccines have been reported for diseases affecting domestic livestock such as infectious keratoconjunctivitis (pinkeye) in cattle (1), footrot in sheep (1,10) and diarrhea in piglets (9) or calves (9). In each of these veterinary examples, the pili vaccine provided protection against challenge by strains expressing the homologous, but not heterologous, pili.

The earliest gonococcal vaccines contained whole organisms and provided little or no protection (11). Recent vaccine development against gonorrhea has focused on purified surface components, in particular, pili (9,11) and the porin protein (P.I or Por) (11). To date, however, only pili have been shown to protect humans from challenge, and this was limited to protection against the homologous strain (12). A denatured form of gonococcal pili (4) has been shown to generate antibodies in mice or guinea pigs which bind to heterologous pili *in vitro*. However, this has not been considered a commercially viable approach because of the difficulty in growing piliated gonococci in

liquid media (a necessity for commercial production) (1). Based on the success of the initial human challenge studies, the gonococcal pilus vaccine was tested in a large, placebo-controlled double blind efficacy trial (12). In this trial, the vaccine failed to protect male volunteers from gonococcal infections. It was postulated that the most likely explanation for this was pilus heterogeneity (12).

Indeed, the antigenic variability of the pilin proteins, both gonococcal and meningococcal, has been repeatedly cited as a major obstacle in the development of pili-based vaccines (3,13,14,15,16,17). It has been suggested that the dominant immune epitope on the assembled, intact pilus is the disulfide loop, which exhibits the greatest sequence variation (17,18). This may account for the failure of the Korean field trial with formalin-treated intact pili from *N. gonorrhoeae* strain Pgh3-2 (12). In addition, the literature contains a number of references in which antisera directed against purified pili, or pilin fragments, bound to denatured (Western blot) or isolated neisserial pili, but did not bind to heterologous pili on bacterial surfaces (16,17,19,20). Whether this is due to antigenic variation or concealment of the epitopes in the assembled pilus has not been completely resolved. This is reinforced by several reports which demonstrated that the only monoclonal antibodies exhibiting functional activity *in vitro* were those which did not bind to heterologous pili (3). It has been shown for the intact pili from *M. bovis* (21) and *D. nodosus* that a protective immune response elicited by pili is possible (10). These vaccines, however, were only able to protect against challenge with bacteria expressing the homologous pili -- not heterologous pili (10,21). The consensus of the

scientific community appears to be that pili-based vaccines, if possible, will protect only against bacteria expressing the homologous pili.

Recombinant expression of assembled pili has been described for a number of organisms, and is dependent on the presence of appropriate transport and assembly genes (22). In *Neisseria*, the genes encoding the proteins involved in pili assembly and export are not found in a single contiguous operon, so this approach was not feasible. Another alternative chosen in European Patent 202,260 B1 was to express type IV pilin genes in a bacterial host which already possessed the proteins required for assembly of a different type IV pilus; e.g., *Pseudomonas aeruginosa* (23). But as reported by Hoyne et al., the expression and assembly of gonococcal pili at the outer membrane of *Pseudomonas* was unstable (24). When the recombinant strain was grown in liquid media in the presence of selective antibiotics, it was overgrown by wild-type, piliated *Pseudomonas*. The authors stated:

> "[T]he compatibility of foreign mePhe [N-methylphenylalanine] pilus production in host strains will depend on the extent of divergence of host and donor pilus assembly systems. The observed instability of PAK/2PfS [*Pseudomonas aeruginosa* strain K/2PfS] expressing gonococcal pilin ... may indicate that the limits of interspecies expression of mePhe pili are being approached in this instance." (24)

Because of this result, the expression of gonococcal pili in *Pseudomonas* was not viewed as a commercially viable approach.

Elleman, Egerton and co-workers described the development of a vaccine against ovine footrot using intact pili from *Dichelobacter nodosus*. Field trials

had demonstrated that intact pili protected against *D. nodosus* strains expressing the homologous pili. This meant that a commercial vaccine would need to contain eight or nine different pili in order to achieve comprehensive protection. In an attempt to make this approach viable, the pilin gene of *D. nodosus* was cloned and expressed in *E. coli* (25). The recombinant pilin protein was found associated with the inner membrane. When a vaccine consisting of sonicated *E. coli* cells expressing the recombinant pilin was tested in a challenge experiment, the recombinant *E. coli* cells generated an antibody titer similar to that seen for purified, native pili (25). However, the agglutination titer induced by the recombinant *E. coli* cell vaccine was significantly lower than that seen for the intact pili (690 vs. 47,800) and below the titer which correlated with protection (5,000-10,000) (25,26).

Following active challenge with *D. nodosus*, the recombinant pilin vaccine failed to show any significant protective activity, in contrast to that seen for the intact pili. Emery and co-workers had demonstrated that denaturation of intact pili abolished the ability of pilin to induce protection in animals (27). In addition, pili dissociated with either detergent (octyl-β-D-glucoside) or low pH (2.2) reduced the effectiveness of the protein to elicit protection against formation of severe lesions following challenge (28). This was despite the fact that the antibody titers were not significantly different between the groups. The authors stated that "there may be one or more epitopes associated with quaternary structure which are disturbed by the treatments". They further stated:

"The failure of the *E. coli* expressed product as a vaccine may result from its physical occlusion in the host cell membranes, although preliminary experiments indicated that this was not the major cause of its ineffectiveness (unpublished data). An alternative explanation for the failure of the *E. coli* expressed product as a vaccine, is that the monomeric prepilin units which are expressed are unable to associate into a native conformation for the appropriate presentation of important epitopes, possibly because of the presence of the leader sequence." (28).

Elleman and co-workers confirmed the importance of the presence of conformational epitopes on the recombinant pilin and that they needed to increase the immune response by a better presentation of the antigen. They proposed two approaches: purification of the protein from the *E. coli* membranes, or expression of protein in *P. aeruginosa* so that it could assemble into filamentous pili on the surface of the cell. The latter approach was preferred because "[t]his should greatly improve the immunogenic properties and simplify the purification of the protein" (25).

Furthermore, Elleman and co-workers also viewed the use of pilin (the subunit protein of pili) as an inferior vaccine candidate to the mature fimbriae (intact pili) for *D. nodosus*:

"Mature fimbriae appear to provoke a more intense and appropriate (*i.e.*, K-agglutinating) immunological reaction than the equivalent dose of fimbrial subunit protein. A serological K-agglutination titre of about 5,000 is generally regarded as the minimum response commensurate with adequate protective immunity against infection

with a given strain of *B. nodosus*. This level of response (and up to an order of magnitude higher) is readily achieved upon vaccination with mature fimbriae, but not the isolated subunit protein, which elicits only poor levels of serum K-agglutinating antibodies" (23).

Additional data suggesting that the fimbrial (pilus) subunit protein is not a viable vaccine candidate was recently reported by Alves et al. (29). When mice were immunized with a polynucleotide vaccine encoding the *E. coli* CFA/I fimbrial adhesin protein (e.g., pilin), the antibodies induced were distinct from those induced by native, intact CFA/I fimbriae. Moreover, these antibodies against the recombinant protein did not exhibit any agglutination activities in contrast to antisera against the native protein.

Despite all the work described above, there is yet to be developed an effective pilus-based gonococcal or meningococcal vaccine. Meningococcal vaccines are limited to those possessing serotype A, C, Y, W135 capsules.

Accordingly, there is a need to identify components for inclusion in vaccines to protect against disease caused by *N. gonorrhoeae* or all serotypes of *N. meningitidis*.

Summary of the Invention

Thus, it is an object of this invention to identify suitable antigenic structures derived from *N. gonorrhoeae* and *N. meningitidis*, respectively, which may constitute viable vaccine candidates against those bacteria. These candidates must induce antibodies which recognize and bind to diverse isolates of the respective pathogenic neisserial organism.

These and other objects of the invention as discussed below are achieved by the cloning and expression of the recombinant pilin protein (rpilin) of each of *N. gonorrhoeae* and *N. meningitidis*.

This invention also relates to the construction of a plasmid which expresses a recombinant meningococcal chimeric class I pilin protein in which the amino-terminal region of the class I meningococcal pilin protein is replaced by the corresponding amino-terminal region of the gonococcal pilin protein. This plasmid expresses significantly higher amounts of the meningococcal chimeric class I rpilin protein than the class I meningococcal rpilin protein expressed from a full-length meningococcal *pilE* gene.

In order to obtain expression of the meningococcal chimeric class I rpilin protein, the chimeric DNA sequence is first inserted into a suitable plasmid vector. A suitable host cell is then transformed or transfected with the plasmid. In an embodiment of this invention, the host cell is an *Escherichia coli* strain. The host cell is then cultured under conditions which permit the expression of said chimeric class I rpilin protein by the host cell.

This invention further relates to the construction of a plasmid which expresses a recombinant meningococcal chimeric class II pilin protein in which the carboxy-terminal region of the class II meningococcal pilin protein is replaced by the corresponding carboxy-terminal region of the gonococcal pilin protein.

In order to obtain expression of the meningococcal chimeric class II rpilin protein, the chimeric DNA sequence is first inserted into a suitable plasmid vector. A suitable host cell is then

transformed or transfected with the plasmid. In an embodiment of this invention, the host cell is an *Escherichia coli* strain. The host cell is then cultured under conditions which permit the expression of said chimeric class II rpilin protein by the host cell.

In another embodiment of this invention, the isolated and purified rpilin protein (either the gonococcal, the meningococcal or chimerics) is used to prepare a vaccine composition which elicits a protective immune response in a mammalian host. The vaccine composition may further comprise an adjuvant, diluent or carrier. Examples of such adjuvants include aluminum hydroxide, aluminum phosphate, MPL™, Stimulon™ QS-21, IL-12 and cholera toxin. The vaccine composition is administered to a mammalian host in an immunogenic amount sufficient to protect the host against disease caused by *N. gonorrhoeae* or *N. meningitidis*.

Brief Description of the Figures

Figure 1 depicts transmission electron micrographs of piliated cells from *N. gonorrhoeae* (strain I756 recA-) incubated with guinea pig antisera directed against gonococcal rpilin (from strain Pgh3-1) (1:50 dilution for 15 minutes), followed by donkey anti-guinea pig IgG conjugated to 12 nm colloidal gold (1:5 dilution for 30 minutes and stained with NanoVan. Figure 1A depicts anti-rpilin guinea pig immune sera (week 6); Figure 1B depicts normal guinea pig sera (week 0); Figure 2C depicts no primary antibody.

Figure 2 depicts the effect of guinea pig antisera directed against gonococcal rpilin (from strain Pgh3-1) on the attachment of piliated *N.*

gonorrhoeae cells (strain I756 recA-) to human cervical cells (ME180 cell line). Figure 2A depicts the inhibition of attachment by guinea pig antisera directed against rpilin (week 6); Figure 2B depicts the inability of normal guinea pig antisera to prevent attachment of piliated gonococcal cells to cervical cells (week 0). Representative sized clumps of bacteria bound to cervical cells are circled in each panel. Each panel shows four different views of the same experimental condition. The guinea pig antisera was diluted 1:10,000 for each panel.

Figure 3 depicts transmission electron micrographs of piliated cells from *N. meningitidis* (strain H355) incubated with guinea pig antisera directed against meningococcal chimeric class I rpilin (from strain H44/76) (1:60 dilution for 30 minutes), followed by donkey anti-guinea pig IgG conjugated to 12 nm colloidal gold (1:5 dilution for 30 minutes) and stained with NanoVan. Figure 3A depicts anti-rpilin guinea pig immune sera (week 6); Figure 3B depicts normal guinea pig sera (week 0); Figure 3C depicts no primary antibody. Cells were fixed before being incubated with antisera.

Detailed Description of the Invention

This invention relates to vaccine compositions comprising a recombinant pilin protein of *N. gonorrhoeae* or *N. meningitidis*. Notwithstanding the teachings of the art discussed above, it was decided to investigate the use of such recombinant pilin proteins expressed in *E. coli*. Surprisingly, these recombinant pilin proteins demonstrated characteristics of vaccine candidates.

The first report describing the cloning of the gonococcal *pilE* gene in *E. coli* was in 1982 (30). Since then, molecular characterization of *pilE* has been performed by numerous laboratories investigating the genetic factors controlling the expression of the pilin protein, transport of the pilin protein, variation in the pilin sequence and the host adherence properties of pili. However, none of the reports described the purification of recombinant pilin protein nor the immune response of the recombinantly expressed pilin protein.

Cloning and expression of the *pilE* gene encoding the gonococcal recombinant pilin protein are described in Example 2 below. Expression was achieved by transforming an *E. coli* strain designated TOP10F' with a plasmid containing the *pilE* gene. Successful cloning and expression was followed by the sequencing of the *pilE* gene to confirm identity with the native sequence. To assist in cloning, a *Nco*I site was introduced, which required modifying one base. As a result, the second amino acid in the seven amino acid long signal peptide was changed from asparagine to aspartic acid.

The plasmid containing the *pilE* gene in Example 2 (designated pPX2000) contains an ampicillin resistance (Amp^R) marker. As described in Example 3, another plasmid was constructed to contain a kanamycin resistance (Kan^R) marker instead of Amp^R. This plasmid, designated pPX2002, after transforming *E. coli* strain TOP10F', expressed the gonococcal rpilin at a level similar to that obtained from pPX2000, which contains an Amp^R marker.

As described in Example 4, a similar procedure was used to construct a plasmid, designated pPX2003, containing the class I *pilE* gene of *N.*

meningitidis. A *Nco*I site (CC ATG G) was introduced spanning the beginning of the gene encoding the signal peptide. This changed the second amino acid residue of the signal peptide from asparagine to aspartic acid (the first residue remained methionine). An Amp^R marker was also included. This construct, after transforming *E. coli* strain TOP10F', expressed class I rpilin of *N. meningitidis*. However, the expression level was significantly lower than that for the gonococcal rpilin obtained from either pPX2000 or pPX2002. Without being bound by theory, this lower expression level may be due to a number of inverted repeats which are present in the recombinant class I *pil*E.

In order to increase the expression of the meningococcal pilin, as described in Example 5, a chimeric plasmid was constructed. The DNA in pPX2003 encoding the first 60 amino acids of the meningococcal class I rpilin is replaced with the equivalent region from the gonococcal DNA in pPX2002. The resulting Amp^R plasmid, designated pPX2004, has the nucleotide sequence set forth in SEQ ID NO:1. The plasmid pPX2004 was used to transform an *E. coli* strain K12 designated TOP10F'. Following induction, there was a significant increase in expression of the chimeric rpilin compared to the amount of meningococcal rpilin expressed from pPX2003. The level of expression of the chimieric construct was comparable to the amount of gonococcal rpilin expressed from pPX2002. The chimeric class I rpilin was 167 amino acids in length (including the signal) (SEQ ID NO:2), which is in accordance with the predicted size.

Samples of the *E. coli* strain K12 designated TOP10F' harboring the recombinant plasmid pPX2004 were deposited on January 27, 1998 by the Applicants with

the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and have been assigned ATCC accession number ATCC 98637.

As described in Example 6, a chimeric plasmid was constructed wherein the 3' end of the class II *pilE* gene of *N. meningitidis* was replaced with the corresponding region from *N. gonorrhoeae*. Specifically, the DNA in pPX8001 encoding the disulfide loop (the last 22 amino acids of the meningococcal class II pilin of *N. meningitidis* strain FAM18) is replaced with a similiar (but larger) region plus additional portions of the carboxy-terminal region totalling 44 amino acids from the gonococcal (*pilE*) DNA from *N. gonorrhoeae* strain Pgh3-1 in pPX2000. The resulting Amp^R plasmid, designated pPX8017, has the nucleotide sequence set forth in SEQ ID NO:3, in which nucleotides 1-378 are from *N. meningitidis* class II and nucleotides 379-510 are from *N. gonorrhoeae*. The plasmid pPX8017 was used to transform the *E. coli* strain K12 designated TOP10F'. Following induction, a chimeric class II rpilin was expressed which was 170 amino acids in length (including the seven amino acid long signal) (SEQ ID NO:4), in which amino acids 1-126 are from *N. meningitidis* class II and amino acids 127-170 are from *N. gonorrhoeae*. This chimeric class II rpilin was in accordance with the predicted size. A *Nco*I site was introduced for cloning considerations, which changed the second amino acid in the signal sequence from lysine to glutamic acid. This change was not expected to have any effect on antigenicity or immunogenicity.

Samples of the *E. coli* strain K12 designated TOP10F' harboring the recombinant plasmid pPX8017 were deposited on April 15, 1999 by the Applicants with the American Type Culture Collection, 10801 University

Boulevard, Manassas, Virginia 20110-2209, U.S.A., and have been assigned ATCC accession number ATCC 207199.

A variety of host cell-vector systems are suitable for use to express the gonococcal, meningococcal and chimeric rpilins used in the vaccines of this invention in addition to those detailed in Examples 2-6. The vector system is compatible with the host cell used. Suitable host cells include bacteria transformed with plasmid DNA, cosmid DNA or bacteriophage DNA; viruses such as vaccinia virus and adenovirus; yeast such as Pichia cells; insect cells such as Sf9 or Sf21 cells; or mammalian cell lines such as Chinese hamster ovary cells; as well as other conventional organisms.

A variety of conventional transcriptional and translational elements can be used for the host cell-vector system. The *pilE* DNA is inserted into an expression system and the promoter and other control elements are ligated into specific sites within the vector, so that when the plasmid vector is inserted into a host cell, the *pilE* DNA can be expressed by the host cell.

The plasmid is introduced into the host cell by transformation, transduction, transfection or infection, depending on the host cell-vector system used. The host cell is then cultured under conditions which permit expression of the rpilin protein by the host cell.

This invention further relates to an isolated and purified DNA sequence comprising a DNA sequence encoding the meningococcal chimeric class I rpilin protein whose amino-terminal region is from the gonococcal *pilE* gene and whose central and carboxy-terminal regions are from the meningococcal *pilE* gene (SEQ ID NO:1). Nucleotides 1-501 in SEQ ID NO:1 encode

the meningococcal chimeric class I rpilin protein prior to processing; nucleotides 22-501 encode the meningococcal chimeric class I rpilin protein after processing to a mature protein. The invention additionally relates to the meningococcal chimeric class I rpilin protein having the amino acid sequence of amino acids 1-167 of SEQ ID NO:2 prior to processing or having the amino acid sequence of amino acids 8-167 of SEQ ID NO:2 after processing to a mature protein. Approximately 10% of the total protein produced by the gonococcal rpilin or the meningococcal chimeric class I rpilin constructs lacks the signal sequence, which has been removed by processing.

This invention further relates to an isolated and purified DNA sequence comprising a DNA sequence encoding the meningococcal chimeric class II rpilin protein whose carboxy-terminal region is from the gonococcal *pilE* gene and whose central and amino-terminal regions are from the meningococcal *pilE* gene (SEQ ID NO:3). Nucleotides 1-510 in SEQ ID NO:3 encode the meningococcal chimeric class II rpilin protein prior to processing; nucleotides 22-510 encode the meningococcal chimeric class II rpilin protein after processing to a mature protein. The invention additionally relates to the meningococcal chimeric class II rpilin protein having the amino acid sequence of amino acids 1-170 of SEQ ID NO:4 prior to processing or having the amino acid sequence of amino acids 8-170 of SEQ ID NO:4 after processing to a mature protein.

In addition to the chimeric DNA sequences contained in pPX2004 and pPX8017 which encode the meningococcal chimeric class I rpilin protein and the meningococcal chimeric class II rpilin protein, respectively, the present invention further comprises DNA sequences which, by virtue of the redundancy of the

genetic code, are biologically equivalent to the sequences which encode for the chimeric rpilin proteins, that is, these other DNA sequences are characterized by nucleotide sequences which differ from those set forth herein, but which encode a protein having the same amino acid sequence as that encoded by the DNA sequence in SEQ ID NO:1 or SEQ ID NO:3.

In particular, the invention contemplates those DNA sequences which are sufficiently duplicative of the sequence of SEQ ID NO:1 or SEQ ID NO:3 so as to permit hybridization therewith under standard high stringency Southern hybridization conditions, such as those described in Sambrook et al. (31).

This invention also comprises DNA sequences which encode amino acid sequences which differ from those of the meningococcal chimeric class I or class II rpilin proteins, but which are biologically equivalent to those described for one of these proteins (SEQ ID NO:2 or SEQ ID NO:4). Such amino acid sequences may be said to be biologically equivalent to those of the chimeric rpilin protein if their sequences differ only by minor deletions from, insertions into or substitutions to the rpilin sequence, such that the tertiary configurations of the sequences are essentially unchanged from those of the rpilin protein.

For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, as well as changes based on similarities of residues in their hydropathic index,

can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal or C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein.

Furthermore, changes in known variable regions are biologically equivalent where the tertiary configurations of the conserved regions are essentially unchanged from those of the rpilin protein. An alternative definition of a biologically equivalent sequence is one that is still capable of generating a cross-reactive immune response. In particular, the meningococcal chimeric class I and II recombinant pilins may be modified by lengthening or shortening the corresponding insertion from the gonococcal pilin, as long as the modified chimeric recombinant pilin is still capable of generating a cross-reactive immune response.

Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of structural and biological activity of the encoded products. Therefore, where the terms "meningococcal chimeric class I rpilin protein" or "meningococcal chimeric class II rpilin protein" are used in either the specification or the claims, it will be understood to encompass all such modifications and variations which result in the production of a biologically equivalent protein.

As described in Example 7, the gonococcal rpilin protein is associated with cellular membranes of the *E. coli* used to express it. A variety of detergents are able to selectively solubilize the rpilin protein from *E. coli*, including Empigen™ BB, Triton™ X-100, reduced Triton™ X-100, octyl-β-D-glucopyranoside (OG), Zwittergent™ 3-10 or 3-14.

Following centrifugation, dialysis and fractionation on a column, the purified rpilin is obtained.

As described in Example 8, the chimeric class I rpilin was isolated and purified by disruption of *E. coli* cells, clarification by centrifugation, filtration, and fractionation on two columns.

As described in Example 9, the meningococcal chimeric class II rpilin was isolated and purified by disruption of *E. coli* cells, clarification by centrifugation, dialysis, and fractionation on two columns.

The purified gonococcal rpilin was subjected to repeated N-terminal sequencing as described in Example 10. Sequencing of the 20-40 amino-terminal residues gave results which agreed with the amino acid sequence deduced from the DNA sequence. The molecular weight of the rpilin (with signal) was determined to be 18,006 daltons by mass spectrometry, which compares well to the predicted mass of 17,981 daltons based on the amino acid content. In contrast, an apparent molecular weight of 68,899 daltons was obtained when the rpilin was subjected to size exclusion column chromatography using detergent. This suggested that the rpilin aggregated. Dialysis of the rpilin against PBS in an effort to remove detergent resulted in material having an apparent molecular weight of 452,349 daltons, as measured by gel filtration. This suggested that it had undergone further aggregation.

As detailed in Example 11, immune sera are obtained by immunizing guinea pigs or mice with the purified gonococcal rpilin. As set forth in Example 12, Western blot analysis showed that antisera against rpilin bound to whole cell lysates from piliated gonococcal cells, while there was no binding seen in non-piliated cell lysates. In contrast, antisera to

the pilin oligomer bound to both piliated and nonpiliated cell lysates.

As detailed in Example 13, when analyzed by ELISA, this pooled antisera against gonococcal rpilin had high endpoint titers for binding to purified gonococcal rpilin protein. Example 13 also details the effects of various adjuvants. When the rpilin was adjuvanted with either MPL™ alone, MPL™ plus aluminum phosphate, or Stimulon™ QS-21, good humoral immune responses in mice were obtained.

As set forth in Example 14, whole cell ELISA showed that antisera against rpilin bound to piliated cells, but not to isogenic non-piliated cells of a particular gonococcal strain.

As described in Example 15, mice were immunized intranasally with gonococcal rpilin with or without native cholera toxin. There was a significant immune response detected in the antigen ELISA from pooled sera generated after the mice were immunized with rpilin in the absence of adjuvant; this response was enhanced by the addition of native cholera toxin. The pooled sera had a low ELISA titer for binding to intact, piliated gonococcal cells; this binding was greatly enhanced when the mice were also immunized with native cholera toxin.

As described in Example 16, immunoelectron microscopy demonstrated that antibodies against rpilin were bound along the length of the pili filaments on the surface of gonococci. This suggested that the antibodies bound to epitopes which would be present on the surface of the bacteria *in vivo*.

Example 17 demonstrates the higher titers obtained for rpilin antisera binding to heterologous piliated bacterial isolates as compared to that obtained for antisera to recombinant pilin oligomer.

The rpilin is converted to rpilin oligomer by dialysis of the rpilin against pH 12 phosphate buffer.

Pili mediate the initial binding of *N. gonorrhoeae* to human mucosal cells. Therefore, if an antigen is able to elicit antibodies which inhibit the attachment of these bacteria to those cells, this would provide evidence that such an antigen is a vaccine candidate.

As discussed in Example 18, guinea pig antisera to rpilin significantly inhibited the binding of gonococci expressing heterologous pili to human cervical epithelial cells. Piliation of gonococci correlates with the infectivity of this bacterium (2,3,32).

These data indicate that the recombinant pilin was able to generate antibodies which bind to diverse pili on intact gonococcal cells and that the antisera exhibits a functional activity (inhibition of bacterial adherence) which would protect immunized human beings against gonococcal colonization and infection (32,33). It has been previously reported that immunization with *E. coli* cells which expressed recombinant pilin from *D. nodosus* were immunogenic (23,25,28), but not protective against challenge. Because of these results, these researchers turned away from the use of recombinant subunit pilin in favor of the assembled pilus. Yet, the data described herein suggest that, following purification, the recombinantly expressed pilin protein induces an immune response which should correlate with protection of humans from gonococcal colonization. Thus, these data support the view that rpilin is a viable vaccine candidate against *N. gonorrhoeae*.

As described in Example 19, the meningococcal chimeric class I rpilin protein was subjected to N-

terminal sequencing. Sequencing of the 35 amino-terminal residues gave results which agreed with the amino acid sequence deduced from the DNA sequence. The molecular weight of the chimeric rpilin (with signal) was determined to be 17,659 daltons by mass spectrometry, which compares well to the predicted mass of 17,676 daltons based on the amino acid content. In contrast, an apparent molecular weight of 69,480 daltons was obtained when the meningococcal chimeric class I rpilin protein was subjected to size exclusion column chromatography using detergent. As with the gonococcal rpilin, this suggested that the meningococcal chimeric class I rpilin protein aggregated.

As detailed in Example 20, when analyzed by ELISA, pooled antisera against the meningococcal chimeric class I rpilin protein had high endpoint titers to both meningococcal class I rpilin protein and to piliated meningococcal cells. As also detailed in Example 20, adjuvants, in particular Stimulon™ QS-21, generated significant responses for the binding of antisera against the meningococcal chimeric class I rpilin protein to both meningococcal class I rpilin protein and to piliated meningococcal cells.

As described in Example 21, immunoelectron microscopy demonstrated that antibodies against the meningococcal chimeric class I rpilin protein were bound along the length of the pili of meningococci. This suggested that the antibodies bound to epitopes which would be present on the surface of the bacteria *in vivo*.

In Example 4, it was shown that antisera directed against gonococcal rpilin recognized and bound to piliated meningococcal cells. In Example 22, it was shown that antisera raised against meningococcal

chimeric class I rpilin protein bound to piliated gonococcal cells.

As described in Example 23, passive immunization of infant rats with guinea pig antisera against meningococcal chimeric class I rpilin protein can help prevent meningococcal bacteremia *in vivo*. There was a significant decrease in the level of colonization in animals who received this immune sera. Furthermore, the immune response generated using recombinantly expressed pilin can protect, *in vivo*, against meningococci which express a heterologous pilin protein.

As described in Example 24, mice were immunized intranasally with meningococcal chimeric class I rpilin with or without cholera toxin, where the cholera toxin is in a mutant form wherein the glutamic acid at amino acid position 29 is replaced by a histidine (CT-CRM, E29H). There was a significant immune response detected in the antigen ELISA from pooled sera generated after the mice were immunized with rpilin in the absence of adjuvant; this response was enhanced by the addition of mutant CT-CRM, E29H cholera toxin.

As described in Example 25, the inhibition of colonization of mouse nasopharynx by a class I strain of *N. meningitidis* was demonstrated in mice immunized subcutaneously with meningococcal chimeric class I rpilin adjuvanted with MPL™.

As described in Example 26, Western blot analysis showed that antisera obtained from guinea pigs immunized with meningococcal chimeric class II rpilin bound to whole cell lysates from piliated meningococcal cells which expressed either class I or class II pilin.

As described in Example 27, antisera elicited against partially purified meningococcal chimeric class

II rpilin bound to meningococcal cells from the homologous bacterial strain.

Taken together, these data support the view that rpilin, in particular the meningococcal chimeric class I and class II rpilin proteins, are viable vaccine candidates against *N. meningitidis*.

The gonococcal rpilin protein is useful in the preparation of vaccines to confer protection to mammals against disease caused by *N. gonorrhoeae*. The meningococcal rpilin protein, the meningococcal chimeric class I rpilin protein and the meningococcal chimeric class II rpilin protein are useful in the preparation of vaccines to confer protection to mammals against disease caused by *N. meningitidis*.

In addition, cross-protection against a different *Neisseria* species is afforded by immunizing with a vaccine containing the gonococcal rpilin protein to confer protection to mammals against disease caused by *N. meningitidis* or by immunizing with a vaccine containing the meningococcal rpilin protein, the meningococcal chimeric class I rpilin protein or the meningococcal chimeric class II rpilin protein to confer protection to mammals against disease caused by *N. gonorrhoeae*.

These vaccine compositions comprise an isolated and purified rpilin protein, wherein the vaccine composition elicits a protective immune response in a mammalian host.

Vaccines containing a rpilin protein may be mixed with immunologically acceptable diluents or carriers in a conventional manner to prepare injectable liquid solutions or suspensions. The level of antibodies elicited by the vaccine may be improved by using certain adjuvants such as Stimulon™ QS-21 (Aquila Biopharmaceuticals, Inc., Framingham, MA), MPL™ (3-O-

deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT), aluminum phosphate, aluminum hydroxide, IL-12 (Genetics Institute, Cambridge, MA) and cholera toxin (either in a wild-type or mutant form, for example wherein the glutamic acid at amino acid position 29 is replaced by another amino acid, preferably a histidine, in accordance with U.S. Provisional Patent Application Number 60/102,430).

The vaccines of this invention are administered by injection in a conventional manner, such as subcutaneous, intraperitoneal or intramuscular injection into humans, as well as by oral, mucosal, intranasal or vaginal administration, to induce an active immune response for protection against disease caused by *N. gonorrhoeae* or *N. meningitidis*. The dosage to be administered is determined by means known to those skilled in the art. Protection may be conferred by a single dose of vaccine, or may require the administration of several booster doses.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

Examples

Standard molecular biology techniques are utilized according to the protocols described in Sambrook et al. (31).

Example 1
Bacteria and Cell Cultures

Bacteria and Culture Conditions

The gonococcal isolates were obtained from Tampa, FL; Ottawa, Canada; Washington, D.C.; Seattle, WA; Rochester, NY; Chapel Hill, NC; and Evanston, IL. The meningococcal isolates were obtained from Chapel Hill, NC; and Bilthoven, Netherlands.

The bacteria were stored in lyophilized form or frozen at -70°C until required. When grown on solid media, the agar plates were incubated in an incubator at 37°C containing a humidified atmosphere and 5% (v/v) CO_2. *N. gonorrhoeae* and *N. meningitidis* were grown on GC medium base (Difco Laboratories, Detroit, MI) without hemoglobin, but supplemented with dextrose (400 mg/L), glutamine (10 mg/L), cocarboxylase (20 μg/L) and ferric nitrate (500 μg/L). Liquid suspension cultures of *N. meningitidis* were grown in the same media which lacked agar in a shaking incubator (70 RPM) at 37°C. In experiments involving culturing of meningococci from mouse nasal tissue homogenates, the bacteria were grown on GC media described above with the following mixture of antibiotics (Difco): colistin sulfate (75 μg/mL), nystatin (125 μg/mL), vancomycin (30 μg/mL) and trimethoprim lactate (50 μg/mL). Piliated gonococci were identified by colony morphology and individual colonies passaged daily in order to maintain the phenotype. The piliation state of meningococcal cells was assessed by transmission electron microscopic examination of samples stained with NanoVan stain (Nanoprobes, Stony Brook, NY) at pH 8 for 30 seconds. *E. coli* were grown on SOB agar which consists of 20 g/L Bacto tryptone (Difco), 5 g/L yeast extract (Difco), 0.6 g/L NaCl, 0.2 g/L KCl and 1% (w/v) agar (pH 7.5) or

in SOB broth to which the agar is not added. For some experiments, the Bacto tryptone was replaced with an equivalent amount of HySoy™ (Sheffield Products, Norwich, NY).

Epithelial cell cultures

ME-180 cell line (ATCC, Beltsville, MD) is an epidermoid carcinoma which was originally derived from a cervical carcinoma. The cells were grown in RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (Sigma, St. Louis, MO), penicillin G (1000 units/mL) (Gibco BRL), L-streptomycin (1 mg/mL) (Gibco BRL) and 2 mM L-glutamine in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. The cells were split every three to four days.

Example 2

Cloning and Expression of Gonococcal *pilE* in *E. coli*

A frozen sample of piliated *N. gonorrhoeae* strain Pgh3-1 was used as the source of the *pilE* DNA in a PCR reaction. The *pilE* gene was amplified using the following primers which recognized the 3' and 5' ends of the complete pilin protein (including the leader sequence): 5' CCC|CGC GCC ATG GAT ACC CTT CAA AAA GGC 3' (PILEFWD) (SEQ ID NO:5) and 5' GGG CCT GGA TCC GTG GGA AAT CAC TTA CCG 3' (PILEREV) (SEQ ID NO:6). The resulting PCR product contained a *Nco*I site at the beginning of the *pilE* coding region and a *Bam*HI site at the end. The *Nco*I site was introduced into the gene because of cloning considerations. This resulted in a change of the second amino acid in the signal sequence from asparagine (AAT) to aspartic acid (GAT). Because amino acid 2 is part of the signal peptide which is

cleaved during normal processing of the mature protein, this change was not expected to have any effect on antigenicity or immunogenicity. The PCR product was cloned into a pCR™II TA cloning vector (Invitrogen, Carlsbad, CA), ligated, and transformed into *E. coli* TOP10F' (Invitrogen). Colonies were selected on 100 μg/mL ampicillin containing plates or on 50 μg/mL kanamycin containing plates. The plasmid DNA was isolated from overnight cultures of these transformants and analyzed by restriction digests using the enzymes *Eco*RI and *Not*I.

Four clones containing an insert of the correct size were submitted for DNA sequence analysis in order to verify the presence of *pil*E PCR DNA fragment. Clone #17, designated pPX1999, was used as the source of the *pil*E gene. Plasmid DNAs from pPX1999 and pTrcHisA (Invitrogen) were digested with *Nco*I and *Bam*HI restriction enzymes, the DNA fragments gel isolated, ligated and transformed into *E. coli* TOP10F'. Ampicillin resistant colonies were selected, the plasmid DNA of the new transformant isolated, and a DNA restriction analysis done using *Bam*HI and *Nco*I restriction enzymes. Two clones with the correct restriction pattern were submitted for DNA sequence analysis. Both clones had the correct DNA sequence and were designated pPX2000.

To test for expression of the recombinant pilin, cultures containing these clones were grown in either shake flasks or a fermentor in SOB plus 100 μg/mL ampicillin and 12 μg/mL tetracycline. When shake flasks were used, *E. coli* were grown in 1L of media until an A_{600} = 0.9-1.0 was obtained. The expression of the recombinant pilin was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and allowing growth to

continue for 1-4 hours, at which point the cells were collected by centrifugation (13,689 x g for 20 minutes at 4°C) and stored at -20°C. For the fermentor, an overnight culture from a plate was used to inoculate a flask containing 500 mL media which was again grown overnight. This liquid culture was then used to inoculate a Biostat B Fermentor (Braun Biotech, Allentown, PA) containing 8.9 L of media. Enhanced growth of the bacteria in the fermentor was obtained when HySoy™-containing media was supplemented with dextrose at a final concentration of 1% (w/v). When the culture reached A_{600} = 1.0, IPTG was added to a final concentration of 1 mM and the cells were allowed to grow for another 1-4 hours before being harvested by centrifugation (13,689 x g for 20 minutes at 4°C). The media was discarded and the cell pellet stored at -20°C. Upon induction with IPTG, expression of rpilin protein increased significantly and reached maximal levels at three to four hours post induction.

Samples of the induced cultures were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The recombinant pilin was visualized using Coomassie blue staining and its identity confirmed by Western blot with a monoclonal antibody specific for gonococcal pili (clone #A33020023, Biospacific, Emeryville, CA).

Example 3

Construction of Gonococcal Recombinant *pilE* Plasmid Containing Kanamycin Resistance Marker

A plasmid was constructed where the Amp^R marker was replaced with a Kan^R marker. Except as noted below, the procedures of Example 2 were used. A PCR reaction was performed on pTrcHisA plasmid DNA

using TrcFXba primer, 5' GGC TCT AGA CTG TCA GAC CAA GTT TAC TC 3' (SEQ ID NO:7), and TrcRXba primer, 5' GGC TCT AGA TTG AAG CAT TTA TCA GGG 3' (SEQ ID NO:8). The underlined sequences code for an *Xba*I restriction site. The approximate 3.5 kb PCR product contained the pTrcHisA DNA minus the ampicillin coding region. Another PCR reaction was performed on pACYC177 plasmid DNA (New England Biolabs, Beverly, MA) using KanFXba primer, 5' GGC TCT AGA TAA ACA GTA ATA CAA GGG G 3' (SEQ ID NO:9), and KanRXba primer, 5' GGC TCT AGA TTA GAA AAA CTC ATC GAG C 3' (SEQ ID NO:10). Again, the underlined sequences code for an *Xba*I restriction site. The approximate 860 bp PCR DNA product contains the kanamycin resistance gene. Both PCR products were purified from an agarose gel, the DNAs were digested with the *Xba*I restriction enzyme, extracted and ligated together. An aliquot of the ligation reaction was transformed into *E. coli* TOP10F' and an aliquot of the transformation mix plated on SOB plates containing 30 μg/mL of kanamycin.

A total of 48 Kan^R colonies were streaked in duplicate onto SOB plates containing either ampicillin or kanamycin. As anticipated, all Kan^R colonies were ampicillin sensitive. Since the cloning design was symmetrical, both orientations of the kanamycin insert were isolated. The kanamycin insert in the same clockwise orientation as the original ampicillin gene was selected for future studies and called pZ564. The DNA region containing the lacIq gene, trc promoter and the multiple cloning site in pZ564 was then replaced with the similar region from the pPX2000 plasmid (which also contained *pil*E) in the following manner: Both pZ564 and pPX2000 were digested with *Sph*I and *Xmn*I restriction enzymes. The approximate 2.2 kb DNA fragment from pPX2000 and the approximate 2.6 kb DNA

fragment from pZ564 were gel purified, ligated together and transformed into *E. coli* TOP10F'. The resulting correct plasmid was called pPX2002.

A similar time course of induction and level of recombinant pilin protein expression was seen when the selection antibiotic was changed to kanamycin from ampicillin.

Example 4

Cloning and Expression of Meningococcal Class I *pilE* in *E. coli*

Because of the highly homologous nature of the DNA and amino acid sequences of gonococcal and meningococcal class I pilins (7), the ability of gonococcal rpilin antisera to bind to pili expressed on meningococcal cells was assessed using the whole cell ELISA. This antisera exhibited a titer of 151,100 for binding to *N. meningitidis* piliated cells from strain H355.

This binding appeared to be directed against the pilin protein, since a Western blot of the whole cell lysate from this strain showed that only a single band co-migrating with pilin bound the antisera (data not shown). A number of other meningococcal strains exhibited lower titers in the whole cell ELISA, but the presence of pili was not confirmed by transmission electron microscopy. Based on these data, it was decided to clone and recombinantly express the class I pilin from *N. meningitidis*. Initially, the same strategy was followed as with the *pilE* from *N. gonorrhoeae*. Except as noted below, the procedures of Example 2 were used.

The class I *pilE* was amplified from the genomic DNA of *N. meningitidis* strain H44/76 using the

following primers: 5′ CCC CGC GCC ATG GAC ACC CTT CAA AAA GGT TTT ACC 3′ (NMFPILE) (SEQ ID NO:11), and 5′ GGG CCT GGA TCC GAG TGG CCG TGG AAA ATC ACT TAC CGC 3′ (NMRPILE) (SEQ ID NO:12). As anticipated, a PCR product of approximately 600 bp DNA was obtained. An aliquot of the PCR reaction product was digested with *Bam*HI and *Nco*I restriction enzymes for insertion into pTrcHisA. The digested DNAs were electrophoresed on an agarose gel and the DNA fragments gel purified. The DNA fragments were then ligated together and transformed into *E. coli* TOP10F′. Miniplasmid prep analysis of ampicillin resistant clones was performed using *Bam*HI and *Nco*I restriction enzymes. Clones expressing the correct restriction digest pattern were called RZ1142 and the plasmid was called pPX2003.

Following induction, the presence of a Coomassie blue stained polypeptide with a molecular weight of approximately 15,000 daltons was observed when a whole cell lysate was analyzed by SDS-PAGE. Analysis of whole cell lysates from four of these clones by SDS-PAGE and Western blot demonstrated the presence of a protein of appropriate mobility and reactivity with polyclonal antisera against the intact pili from *N. gonorrhoeae* strain LB2. The purified class I rpilin was 167 amino acids in length. However, the level of expression of this recombinant pilin was significantly lower than that obtained with either pPX2000 or pPX2002 grown under the same conditions. An analysis of the DNA sequence in the recombinant class I *pil*E showed that there were a number of inverted repeats which might explain the low level of expression of this protein in *E. coli*.

Example 5

Construction and Expression of a Gonococcal and Meningococcal Class I Chimeric *pilE* in *E. coli*

In order to increase the expression of the meningococcal pilin protein, the DNA encoding the first 60 amino acids in pPX2003 (the meningococcal class I *pilE* construct described in Example 4) was replaced with the equivalent region from pPX2002 (the gonococcal *pilE* construct described in Example 3, including the seven amino acid signal peptide) (SEQ ID NO:4). Except as noted below, the procedures of Example 2 were followed.

The conserved 5′ terminal region of the meningococcal *pilE* gene was replaced by the same region from *N. gonorrhoeae* strain Pgh3-1 in the following manner. A *Bsm*BI site was introduced into the meningococcal *pilE* gene as follows: DNA was PCR amplified from pPX2003 using the following primers: 5′ CCG GCG CGT CTC TCA CGG CGA ATG GCC CGG C 3′ (CL-1ESPF) (SEQ ID NO:13) and 5′ GGG CCT GGA TCC GAG TGG CCG TGG AAA ATC ACT TAC CGC 3′ (NMRPILE) (SEQ ID NO:14) and Taq DNA polymerase. The expected PCR DNA product was cloned directly into pCR2.1 (Invitrogen) and transformed into TOP10F′ cells and the resulting plasmid was designated pZ578. A *Bsm*BI site was then introduced into the gonococcal *pilE* by the following method. Using the primers 5′ GCA TAA TTC GTG TCG CTC AAG GCG C 3′ (TRCUPFW) (SEQ ID NO:15) and 5′ GCC GCG CGT CTC CCG TGA TTC AGG TAA TAC TCG G 3′ (PILEESPR) (SEQ ID NO:16) and Pfu DNA polymerase, the 5′ end of the *pilE* gene from pPX2000 was PCR amplified. The resulting gonococcal PCR product and pZ578 were then digested with *Bsm*BI and ligated together. The ligated DNAs were

then PCR amplified using 5′ GCA TAA TTC GTG TCG CTC AAG GCG C 3′ (TRCUPFW) (SEQ ID NO:17) and 5′ GGG CCT GGA TCC GAG TGG CCG TGG AAA ATC ACT TAC CGC 3′ (NMRPILE) (SEQ ID NO:18) primers.

The DNA PCR product was of the predicted size (approximately 850 bp) and was digested with *Nco*I and *Bam*HI to yield an approximately 600 bp fragment. This fragment was gel isolated and cloned directly into *Nco*I- and *Bam*HI-cut pPX2000 vector, replacing the gonococcal *pil*E gene. The resulting plasmid, which was ampicillin resistant, was labeled pPX2004 and used to transform TOP10F′. Analysis of this transformant demonstrated the presence of the desired chimeric *pil*E DNA. Following induction with IPTG, there was a significant increase in the amount of the meningococcal chimeric class I rpilin construct expressed compared to the amount of meningococcal rpilin expressed from pPX2003. Using the extraction with 1% octyl-β-D-glucopyranoside (OG) and purification protocol (TMAE Fractogel™ column in 10 mM Tris™, pH 8.5 with 0.1% (w/v) Zwittergent™ 3-14) described in Example 7 for the recombinant gonococcal pilin, highly purified meningococcal chimeric class I rpilin protein was obtained (yield approximately 5 mg/gram cell wet weight). This material was greater than 90% pure when analyzed by SDS-PAGE and laser densitometry. SDS-PAGE demonstrated the presence of a major band of approximately 15,000 daltons in size. The meningococcal chimeric class I rpilin protein was also 167 amino acids in length, and includes the signal sequence of seven amino acids as demonstrated by sequencing of the amino-terminal 36 residues of the purified protein.

Example 6

Construction and Expression of a Gonococcal and Meningococcal Class II Chimeric *pilE* in *E. coli*

The initial cloning of the meningococcal class II *pilE* involved isolation of chromosomal DNA from piliated *N. meningitidis* strain FAM18 cells and amplifying the classII *pilE* DNA in a PCR reaction. The class II *pilE* gene was amplified using the following primers which recognized the 3' and 5' ends of the complete pilin protein (including the leader sequence): 5' GCG GCC GCC ATG GAA GCA ATC CAA AAA GGT TTC ACC C 3' (PILE2FWD) (SEQ ID NO:19) and 5' GCG GCC GGA TCC GGT CAT TGT CCT TAT TTG GTG CGG C 3' (PILE2REV) (SEQ ID NO:23). In a similar strategy as in Example 2, the resulting PCR product contained an *Nco*I site at the beginning of the *pilE* coding region and a *Bam*HI site at the end. The *Nco*I site was introduced into the gene because of cloning considerations. This resulted in a change of the second amino acid in the signal sequence from lysine (AAA) to glutamic acid (GAA). As stated previously, this change was not expected to have any effect on antigenicity or immunogenicity. The PCR product was cloned into a pCR2.1 cloning vector (Invitrogen), ligated, and transformed into *E. coli* TOP10F'. Colonies were selected on 100 μg/mL ampicillin-containing plates or 50 μg/ml kanamycin plates. The plasmid DNA was isolated from overnight cultures of these transformants and analyzed by restriction digests using the enzyme *Eco*RI.

Clone #8, designated pPX8001, was used as the source of the *pilE* gene. Plasmid DNAs from pPX8001 and pTrcHisC (Invitrogen) were each digested with *Nco*I and *Bam*HI restriction enzymes, and the resulting DNA fragments were gel isolated, ligated and transformed

into *E. coli* TOP10F'. Following selection of ampicillin resistant colonies, the plasmid DNA of the new transformants were isolated, and a DNA restriction analysis performed using *Bam*HI and *Nco*I restriction enzymes. Two clones with the correct restriction pattern were submitted for DNA sequence analysis. Both clones had the correct DNA sequence, designated as pPX8002.

To test for expression of the recombinant class II pilin, 10 mL cultures containing these clones, pPX8002, were grown in 50 mL tubes in SOB containing 100 μg/mL ampicillin and 12 μg/mL tetracycline, to an A_{600} = 1.0. Expression of the recombinant protein was induced by adding IPTG to final concentration of 1 mM and the culture was continued for three hours. When a whole cell lysate of the induced cells was separated by SDS-PAGE and stained with Coomassie blue, no new (induced) band was detected. This suggested that the FAM18 *pilE* gene product was expressed at levels lower than those discernable with Coomassie blue. When the FAM18 *pilE* was cloned into the pET17b plasmid and transformed into *E. coli* BL21(DE3)pLysS with or without the *pilE* signal sequence, no significant expression of recombinant protein was detected. Similar results were obtained when the class II *pilE* gene from two other strains of *N. meningitidis* (NmB, 2996) were cloned into the same pTrcHis plasmid and TOP10F' expression system.

Specifically, the strains NMB and 2996 were also determined to be expressing the class II pilin, based on PCR and sequencing data. The *pilE* gene was amplified from several *N. meningitidis* strains using a class I set of primers (NMFPILE and NMRPILE) and a class II set of primers (PILE2FWD and PILE2REV) in separate reactions with either chromosomal DNA or cells as the template. PCR products were cloned into

pTrcHisC and sequenced, or were sequenced directly. Alignments of sequences were carried out; sequences similar to those from the H44/76 strain were classified as class I, while sequences similar to those from the FAM18 strain were classified as class II. Sequences were not obtained for all the strains amplified. A preliminary classification was also made based on PCR data. Class I strains were those which gave a correct size PCR product with class I primers but not with class II primers, while class II strains were those which gave a correct size PCR product with class II primers but not with class I primers.

Based on the experience with the meningococcal class I pilin, the region encoding the first 60 amino acids (the conserved amino-terminal region) of the class II *pilE* were replaced with the corresponding region from *N. gonorrhoeae* strain Pgh3-1. Expression of the resulting chimeric *pilE* was investigated in a number of *E. coli* expression strains using a variety of promoters. The strains studied included: PR13 (Rnase deficient), BL21 (protease deficient), KS474 (deficient in periplasmic protease), AD494 (Novagen, which allows disulfide bond formation in cytoplasm) and three strains of TOPP (Stratagene, non-K12 strains useful for hard to express proteins). In all cases, no recombinant protein was detected in Coomassie blue- stained SDS-PAGE. An alternative expression plasmid pET17b (which includes a T7 promoter) was investigated with similar results.

It should be noted that the native class II *pilE* gene sequence in pPX8002 ends at base 447. The DNA sequence found downstream (3') from the native meningococcal classII *pilE* termination site, nucleotides 447 to 519, contains an inverted repeat which might form a stem and loop structure. Because

stem and loop structures can be effective terminators of transcription, it was postulated that the omission of this additional 3' sequence (74 bases) in pPX8002 might affect the transcription of the chimeric class II *pilE* message in *E. coli*. Therefore, all subsequent cloning restored this downstream 3' end sequence.

A systematic replacement of various portions of the class II *pilE* gene from the *N. meningitidis* strain with the corresponding regions of the *pilE* gene from *N. gonorrhoeae* strain Pgh3-1 was undertaken in order to identify regions that inhibited expression. Replacement regions started at the 5' or 3' ends and were made progressively larger. Double replacements of 5' and 3' ends were also constructed until an internal region of only 84 nucleotides from the native *pilE* class II remained. This region was also replaced, resulting in the reconstruction of the rGC and, as expected, this clone expressed rpilin at similar Coomassie blue stained levels as pPX2000. The following regions (listed by nucleotide numbers) of the FAM18 *pilE* gene were replaced with the corresponding regions from Pgh3-1 (listed in parentheses): single region replacements were 1-108 (1-108), 1-181 (1-181), 1-294 (1-282), 439-499 (478-553), 379-519 (367-553), 295-519 (283-553), 295-378 (283-366); double region replacements were 1-294 (1-282) & 379-519 (367-553), 1-181 (1-181) & 439-499 (478-553), 1-181 (1-181) & 379-519 (367-553), 1-294 (1-282) & 439-499 (478-553).

When these constructs were expressed in *E. coli* TOP10F' using the pTrcHis expression system, two constructs produced recombinant protein at levels detectable by Coomassie blue: the first containing the replacement of nucleotides 379-519 (which comprises the disulfide loop and 3' extension); and the second containing the replacement of both nucleotides 1-181

(which comprises the conserved 5′ region) & 379-519 (which comprises the disulfide loop and 3′ extension). Because replacement of the 5′ region alone did not lead to expression of recombinant protein and because the first construct retained most of the native meningococcal pilin sequence, this construct (nucleotides 379-519) was selected for further investigation. While the amino acid sequence of the meningococcaal and gonococcal proteins are significantly different in this region, it is well documented (17,18) that the disulfide loop undergoes significant antigenic variation. Therefore, any immune response directed against this region (e.g., the disulfide loop) would exhibit minimal cross-reactivity among meningococcal strains. Lastly, because the gonococcal insert is nearly twice the size of the meningococcal disulfide loop (39 residues versus 18 residues), the resulting chimeric protein migrates on an SDS-PAGE gel with an apparent molecular weight of approximately 19,000 daltons.

The construction of this chimeric gene was carried out in the following manner. The 5′ fragment was obtained by amplifying pPX8002 (FAM18 class II *pilE*) with the following primers: 5′ GCG GCC GCC ATG GAA GCA ATC CAA AAA GGT TTC ACC C 3′ (PILE2FWD) (SEQ ID NO:19) and 5′ GCC GCG CGT CTC CGA ACC GGA GTT TTG TTT GCC 3′ (REV-CYS) (SEQ ID NO:20). The gonococcal disulfide loop (i.e., the 3′ end of the gonococcal gene) was amplified from pPX2000 using primers 5′ CCG GGC CGT CTC GGT TCG GTA AAA TGG TTC TGC 3′ (FWD-CYS) (SEQ ID NO:21) and 5′ GGG CCT GGA TCC GTG GGA AAT CAC TTA CCG 3′ (PILEREV) (SEQ ID NO:22). The resulting PCR products were each purified, digested separately with restriction enzyme *Bsm*BI, then ligated to form the full length chimeric *pilE*, which was amplified using primers

PILE2FWD and PILEREV. This PCR product was digested with restriction enzymes *Nco*I and *Bam*HI, ligated into a similarly restricted pTrcHisC vector and transformed into TOP10F' competent cells.

Transformants were cultured and analyzed using the restriction enzymes *Nco*I and *Bam*HI. Four clones with the right sized insert were analyzed with restriction enzyme *Stu*I. Of these, three provided the correct restriction map. Two of the three clones with the correct restriction pattern were sequenced. Clone #5 had the correct DNA sequence and was designated as pPX8017. This clone contains the nucleotide sequence set forth in SEQ ID NO:3, in which nucleotides 1-378 are from *N. meningitidis* and nucleotides 379-510 are from *N. gonorrhoeae*.

Expression was checked with 10mL cultures in 50mL tubes. Cells were grown in SOB supplemented with 100 µg/ml ampicillin and 12 µg/ml tetracycline until A_{600} approximately 1.0. A culture was induced by the addition of IPTG to a final concentration of 1 mM. Growth was allowed to continue for 3-4 hours, at which point the cells were collected by centrifugation (13,689 x g for 20 minutes at 4°C) and stored at -20°C. For the fermentor, an overnight culture from a plate or frozen stock was used to inoculate a flask containing 500 mL media which was again grown overnight. This liquid culture was then used to inoculate a Biostat B Fermentor (Braun Biotech, Allentown, PA) containing 8.9 L of media. Enhanced growth of the bacteria in the fermentor was obtained when HySoy™-containing media was supplemented with dextrose at a final concentration of 1% (w/v). When the culture reached A_{600} = 4.0-6.0, IPTG was added to a final concentration of 1 mM and the cells were allowed to grow for another 2-4 hours before being harvested by centrifugation (13,689 x g for 20

minutes at 4°C). The media was discarded and the cell pellet stored at -20°C. Upon induction with IPTG, expression of chimeric class II rpilin protein increased significantly.

Samples of the induced cultures were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The recombinant meningococcal chimeric class II pilin was visualized using Coomassie blue staining (apparent molecular weight of approximately 19,000 daltons) and its identity confirmed by Western blot with a polyclonal antisera directed against a gonococcal peptide (Glu Ala Ile Leu Leu Ala Glu Gly Gln Lys Ser Ala Val Thr Glu Tyr Tyr Leu Asn His Gly Lys) (SEQ ID NO:24), which is located in the conserved region of the amino terminus of the class II pilin protein. The meningococcal chimeric class II rpilin was 170 amino acids in length (including the signal) (SEQ ID NO:4), in which amino acids 1-126 are from *N. meningitidis* and amino acids 127-170 are from *N. gonorrhoeae*.

Example 7

Isolation and Purification of Recombinant Gonococcal Pilin from *E. coli*

The following procedure was used to purify the recombinant gonococcal pilin obtained in Examples 2 and 3 above. This procedure is also used to purify the meningococcal recombinant pilin obtained in Example 4, and was used initially to purify the meningococcal chimeric class I rpilin protein obtained in Example 5, above. Subsequently, the isolation procedure for the meningococcal chimeric class I rpilin protein was modified as described in Example 8 below.

Sub-cellular fractionation of the *E. coli* expressing rpilin demonstrated that the protein was associated with the cellular membranes, most likely the inner membrane, based on the ability of 1% (v/v) Triton™ X-100 to solubilize this protein. When an attempt was made to remove contaminating *E. coli* proteins in the presence of 0.05-0.1% (v/v) Triton™ X-100, it was discovered that, below pH 9.5, the rpilin did not bind consistently to an ion exchange column. Therefore, the ability of a number of detergents to selectively solubilize the rpilin protein from *E. coli* membrane preparations was examined.

The cell pellet (approx. 5 g wet weight) from 1 L of culture was thawed by adding 30 mL of 10 mM Hepes (pH 7.2) (Research Organics, Cleveland, OH), 1 mM EDTA and the cells broken using a Microfluidizer cell homogenizer (Microfluidics International Corp., Newton, MA). The lysate was clarified by centrifugation (12,000 x g for 10 minutes) and the membranes pelleted (288,652 x g for one hour). The membranes were resuspended in 33 mL of 10 mM Hepes (pH 7.4), 1 mM $MgCl_2$ and extracted with one of the following detergents: (a) Triton™ X-100 (TX100) (Calbiochem-Novabiochem International, San Diego, CA), (b) reduced Triton™ X-100 (Calbiochem), (c) octyl-β-D-glucopyranoside (OG) (Calbiochem), (d) Zwittergent™ 3-8 (Z3-8) (Calbiochem), (e) Zwittergent™ 3-10 (Z3-10) (Calbiochem), (f) Zwittergent™ 3-12 (Z3-12) (Calbiochem), (g) Zwittergent™ 3-14 (Z3-14) (Calbiochem), (h) Empigen BB™ (Calbiochem) or (i) Tween™ 80 (ICN, Cleveland, OH) for one hour at room temperature.

Empigen™ BB (1% v/v), Zwittergent™ 3-10 (1% w/v), reduced Triton™ X-100 (1% v/v), octyl glucoside (1% w/v) with Zwittergent™ 3-10 (1% w/v) or 3-14 (0.1%

w/v) each selectively extracted the recombinant pilin protein with minimal contamination with the *E. coli* proteins. Zwittergent™ 3-12, even at 0.1% (w/v), solubilized both the recombinant protein and a significant number of *E. coli* proteins. Tween™ 80 did not extract the recombinant protein at any tested concentration (0.1-1% v/v).

The solubilized proteins were separated from insoluble membrane material by centrifugation (288,652 x g for one hour). The supernatant (containing rpilin) was dialyzed overnight at 4°C against 10 mM Tris™ (pH 8.5) containing one of the following non-ionic detergents: (a) 0.1% (w/v) Zwittergent™ 3-14, (b) 1% (w/v) Zwittergent™ 3-10 or (c) 1% (w/v) OG. The dialyzed material was fractionated on a Fractogel™ EMD TMAE-650(S) (EM Separations Technology, Wakefield, RI) column equilibrated in 10 mM Tris™ (pH 8.5) and the respective detergent. The bound protein was eluted with a linear gradient of 0 to 0.2 M NaCl in 10 mM Tris™ (pH 8.5) containing the appropriate detergent. Fractions containing rpilin were pooled, analyzed for purity and protein content. Occasionally, to increase the purity of the rpilin, the pooled material was dialyzed against the starting buffer and fractionated a second time on the TMAE column.

The rpilin, which was selectively eluted from the column, was highly purified, as judged by laser densitometric analysis of a Coomassie blue stained SDS-PAGE (>90% homogeneous). Similar results were obtained when the extraction and column chromatography were done with 1% (w/v) Zwittergent™ 3-10, 1% (w/v) OG or 0.1% (w/v) Zwittergent™ 3-14. The yield of rpilin, which was a significant proportion of the total *E. coli* protein, was approximately 10 mg/L of culture grown in 1.5 L shake flasks with SOB media. When the

recombinant *E. coli* (containing pPX2002) were grown in a fermentor using HySoy™ based media, the yield of purified rpilin increased to approximately 30 mg/L of culture, which corresponds to seven mg rpilin per gram of cell mass. When 1% dextrose was included in the fermentor, the overall yield of rpilin increased to approximately 100 mg/L.

The purified rpilin was dialyzed against 10 mM sodium phosphate, 140 mM NaCl (pH 7) (PBS) containing 0.05% (w/v) Z3-14, sterile filtered and stored at 4°C or frozen at -20°C.

Example 8

Isolation and Purification of Meningococcal Chimeric Class I rpilin from *E. coli*

Large scale cultures of *E. coli* cells containing pPX2004 were grown in a Biostat B Fermentor as described in Example 2. Bacterial cells (approximately 88 grams wet weight of *E. coli* pPX2004) were resuspended in 440 mL of 10 mM Hepes, 1 mM EDTA (pH 7.5) and disrupted using a Microfluidizer Model 110Y (Microfluidics Corp., Newton, MA). The disrupted cells were clarified by centrifugation at 6,084 x g for 20 minutes at 10°C. The supernatant was collected and the membrane fraction isolated by centrifugation at 205,471 x g for 1 hour at 10°C. The pellet was resuspended by homogenization in 220 mL of 10mM Hepes, 1 mM $MgCl_2$, 1% (w/v) octyl-β-D-glucopyranoside (pH 7.5) and stirred for 90 minutes at room temperature. The suspension was centrifuged at 205,471 x g for one hour at 10°C. Following centrifugation, the supernatant, which contained the solubilized chimeric class I rpilin, was filtered through a 0.22μ Nalgene vacuum filter and stored at 4°C. The pH of the octylglucoside

extract was adjusted to pH 8.5 with concentrated NaOH and subsequently loaded onto a 200 mL TMAE Fractogel™ column (EM Separations Technology, Gibbstown, NJ) equilibrated with 25 mM Tris™, 0.1% (w/v) Zwittergent™ 3-14 (pH 8.5). Unbound protein was washed through the column with an additional 400 mL of the equilibration buffer. The rpilin was eluted using a linear NaCl gradient (0-0.2 M NaCl) in 25 mM Tris™, 0.1% (w/v) Zwittergent™ 3-14 (pH 8.5) over 10 column volumes at a flow rate of 10.0 mL/minute. Fractions containing the chimeric class I rpilin were pooled and diluted 1:1 with dH_2O and loaded onto a 100 mL 40 μm ceramic hydroxyapatite column (Bio-Rad, Hercules, CA) equilibrated with 10 mM $NaPO_4$, 0.1% (w/v) Zwittergent™ 3-14 (pH 6). Unbound protein was washed through the column with an additional 200 mL of equilibration buffer. The chimeric class I rpilin was eluted using a linear $NaPO_4$ gradient (10-150 mM NaPO4) containing 0.1% (w/v) Zwittergent™ 3-14 over 10 column volumes at a flow rate of 5.0 mL/minute. Fractions were screened by SDS-PAGE analysis and those containing the chimeric class I rpilin were pooled. The purified material was at least 95% pure, as determined by laser densitometry of Coomassie blue-stained gels. The yield of purified chimeric class I rpilin was approximately 35 mg/g wet weight cells.

Example 9

Isolation and Purification of Meningococcal Chimeric Class II rpilin from *E. coli*

All steps were performed at room temperature unless specified. Frozen *E. coli* cells were resuspended in 10 ml of 10 mM Hepes (pH 7.2), 1 mM EDTA

per gram of cells and homogenized using a Microfluidizer cell homogenizer to disrupt the cells. The cell lysate was clarified by centrifugation at 13,689 x g for 30 minutes. The resulting supernantant was then centrifuged at 388,024 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet containing the membranes was frozen at -20°C overnight. The membrane pellet was resuspended in 9 mL/tube of 10 mM Hepes (pH 7.2), 1 mM $MgCl_2$ and extracted with 1% (w/v) Zwittergent™ 3-16 (Calbiochem) for one hour. The suspension was centrifuged at 388,024 x g for 30 minutes and the resulting pellet was extracted again with Zwittergent™ 3-16 as described above. Following centrifugation (388,024 x g for 30 minutes), the pellet was resuspended into 9 mL of 50 mM Tris™ (pH 8.0), 5 mM EDTA and extracted with 1% (w/v) N-laurylsarcosyl (Sigma) with gentle agitation overnight at room temperature. This resulted in the solubilzation of the meningococcal chimeric class II rpilin. The insoluble material was removed by centrifugation (388,024 x g for 30 minutes) and discarded. Zwittergent™ 3-14 was added to the supernantant, which contained the meningococcal chimeric class II rpilin, to a final concentration of 1% (w/v) and the material was dialyzed overnight against 50 mM Tris™ (pH 8.0), 10 mM EDTA, 1% Zwittergent™ 3-14. An aliquot (1 mL, 1.3mg protein) of the dialyzed material was then passed over a Mono-Q™ (Pharmacia, Piscataway, NJ) column (5x10 mm) which was equilibrated in 50 mM Tris™ (pH 8.0), 10 mM EDTA, 1% (w/v) Zwittergent™ 3-14 at a flow rate of 0.5 mL/minute. The unbound material containing the chimeric class II rpilin was pooled and dialyzed overnight against 10 mM $NaPO_4$ (pH 6.8), 1% (w/v) Zwittergent™ 3-14. This material was approximately

80% pure and was used in the studies described in Examples 26 and 27 below. Further purification of this material was obtained by passing it over a 1 mL hydroxyapatite column (Bio-Rad) which was equilibrated in 10 mM $NaPO_4$ (pH 6.8), 1% (w/v) Zwittergent™ 3-14. The purified meningococcal chimeric class II protein was eluted with a linear gradient of 0-0.5M $NaPO_4$ containing 1% (w/v) Zwittergent™ 3-14. Fractions were screened for chimeric class II rpilin by SDS-PAGE, using gels which were stained with either Coomassie blue or silver. Both analyses demonstrated the presence of a single polypeptide band which had a molecular weight of approximately 19,000 daltons. This material was shown to be greater than 95% pure by laser densitometric analysis of the polyacrylamide gels.

Example 10
Analytical Methods for Gonococcal rpilin

Protein content was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using BSA as the standard. The purity of protein preparations was determined by Coomassie brilliant blue stained polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and analyzed by laser denistometry with a Personal Densitometer SI (Molecular Devices). The identity of pilin in the preparations was confirmed by Western blotting using the monoclonal antibody described in Example 2, which is raised against purified pili from *N. gonorrhoeae* strain P9 (Biospacific). The N-terminal sequences of the pilin proteins were determined using an Applied Biosystems 477A Protein Sequencer. Two sequences were often detected when the purified rpilin was submitted for N-terminal sequencing. The major sequence represented

the complete pilin protein, including the seven amino acid leader sequence. The minor sequence, comprising 10-20% of the sample, was rpilin protein in which the leader sequence was missing and the sequence started at phenylalanine, the N-terminal residue of the mature gonococcal pilin protein. For both rpilin protein forms, sequencing of the amino-terminal residues gave results which agreed with the sequence deduced from the DNA sequence.

The mass of recombinant pilin was determined by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a Finnagan MAT Lasermat™ 2000 (San Jose, CA). The instrument was calibrated with horse myoglobin to within 0.01% of its expected mass of 16,951.5 daltons. Recombinant pilin was mixed with an equal volume of a cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 70:30 acetonitrile : 0.1% (v/v) trifluoroacetic acid/water). An aliquot (1 μL) of this mixture was deposited on a sample stage, allowed to air dry, and subjected to MALDI-TOF mass spectrometry analysis. Data from 15 runs (each run representing a sum of 10 shots) were averaged to determine the mass of rpilin. The molecular weight of the rpilin (with signal) was determined to 18,001 daltons, which compares well to the predicted mass of 17,981 daltons based on the amino acid content. A minor peak with a mass of 17,232 daltons (average) was detected in each lot. The difference in molecular weights of the two forms of the recombinant pilin (769 daltons) is ascribed to the loss of the first six amino acids of the leader sequence (Met Asp Thr Leu Gln Lys) (SEQ ID NO:2, amino acids 1-6) which has a mass of 774 daltons.

A very different apparent molecular weight of the rpilin was obtained by size exclusion column

chromatography using an analytical Superose™ 12 column (Pharmacia, Piscataway, NJ) equilibrated in PBS containing 0.05% (w/v) Zwittergent™ 3-14. Under these conditions, the protein eluted at a position corresponding to a molecular weight of 68,899. This suggested that the recombinant protein aggregated. However, the elution of a protein from a size exclusion column can be greatly influenced by the shape of the protein. Results from velocity sedimentation centrifugation experiments demonstrated that the recombinant pilin had a molecular weight in solution of approximately 45,000 daltons. In an attempt to remove the detergent (Zwittergent™ 3-14), the recombinant protein was dialyzed extensively against PBS alone. The dialyzed recombinant protein appeared soluble and was not pelleted by high speed centrifugation (122,000 x g for one hour). No attempt was made to verify the complete removal of the detergent from the recombinant protein. Analysis of this material by gel filtration in PBS indicated that the protein had an apparent molecular weight of 452,349 daltons. This suggested that it had undergone further aggregation. The number of subunits in either aggregate has not been determined.

The value of 452,349 must be considered an estimate, because the protein may still be in a micelle, as it is unknown if the detergent was completely removed from the sample. Given the fact that rpilin is diluted approximately 15-30 fold when formulated as a vaccine, it appears likely that the rpilin in the vaccine will have an apparent molecular weight of approximately 450 kD.

Example 11
Preparation of Immune Sera from rpilin

Immunogenicity studies were performed using guinea pigs (female, 200 g) immunized subcutaneously (s.c.) with 20 μg of purified gonococcal rpilin protein mixed with an adjuvant. The adjuvants studied were: (a) Stimulon™ QS-21 (25 μg/dose) in PBS (pH 6); (b) aluminum phosphate (Lederle Laboratories, Pearl River, NY, 100 μg/dose) in PBS (pH 7); or (c) PBS (pH 7) only. Initially, the animals were immunized on weeks 0, 4 and 8 and sera were obtained on weeks 0, 4, 6 and 10. Analysis of the time course of the immune response demonstrated that giving a third vaccination at week 8 did not boost the immune response and, therefore, later studies with the recombinant pilin were terminated at the week 6 bleed.

In order to investigate the ability of adjuvants to modulate the immune response of gonococcal rpilin, mice (female, 8 weeks old, 5 or 10 animals per group) were immunized subcutaneously with 1-10 μg of purified protein on weeks 0, 4 and 6 and sera were obtained on weeks 0, 4, 6 and 8. Vaginal lavages were done at week 8 by instilling RPMI 1640 (75 μL) into the vagina and aspirating 3-4 times. The lavage fluids from each group were pooled together and 50 μL of fetal bovine serum was added to each pool.

For the meningococcal chimeric class I rpilin protein, the mice received immunizations on week 0 and 4 only and sera was obtained on weeks 0, 4 and 6. For all rpilin parenteral immunogenicity studies in mice, the following adjuvants were studied: (a) Stimulon™ QS-21 (25 μg/dose) in PBS (pH 6); (b) aluminum phosphate (100 μg/dose) in PBS (pH 7); (c) MPL™ (50 μg/dose) in PBS (pH 7); (d) aluminum phosphate (100

μg/dose) and MPL™ (50 μg/dose) in PBS (pH 7); or PBS (pH 7) only.

For the meningococcal chimeric class II rpilin protein, the guinea pigs received immunizations of 20 μg protein adjuvanted with Stimulon™ QS-21 (25 μg/dose) in PBS (pH 6) on week 0 and 4 only and sera was obtained on weeks 0, 4 and 6.

The ability of the recombinantly expressed pilins (either gonococcal or meningococcal chimeric class I) to induce a mucosal immune response was assessed by immunizing mice intranasally with (a) 1 or 10 μg of gonococcal rpilin in 2.5 μL of saline with or without 1 μg of native cholera toxin, or (b) 5 μg of chimeric class I rpilin diluted in 10 μL of PBS (pH 7), with or without 1 μg of mutant CT-CRM, E29H cholera toxin. The immunizations were given on weeks 0, 2 and 3.

Example 12

Western Blot Analysis of the Immune Response Against Gonococcal rpilin

The purified gonococcal rpilin was used to immunize guinea pigs following the protocol described in Example 11. The antisera derived from guinea pigs immunized with gonococcal rpilin were analyzed first by Western blots (data not shown). These blots demonstrated that the antisera against gonococcal rpilin recognized a band corresponding to pilin in whole cell lysate from piliated gonococcal cells; there was no staining seen in non-piliated cell lysate from the same gonococcal strain.

Comparative data were obtained from antisera from guinea pigs immunized with the gonococcal pilin oligomer. Pilin oligomer was obtained by dissociation

of intact pili as previously described (4). Briefly, this involved dialysis of intact pili against 37 mM sodium phosphate (pH 12) for 48 hours at 4°C, followed by dialysis against 50 mM Tris™, 145 mM NaCl (pH 8.0). The pilin oligomers were then clarified by centrifugation (100,000 x g for one hour). Following centrifugation, the pilin oligomers remained in the supernatant. In comparison to antisera against gonococcal rpilin, pilin oligomer antisera, while binding to pilin in the piliated cell lysate, also bound to a number of other bands in the lysates from both piliated and non-piliated cells (data not shown). These bands represent contaminants in the pilin oligomer preparation and are presumed to be not associated with pili.

Example 13
Recombinant Gonococcal Pilin ELISA

The endpoint titers against purified proteins or bacterial cells were determined by ELISA. In all ELISA procedures, incubations were for one hour at room temperature, unless otherwise specified. Endpoint titers were defined as the extrapolated dilution at which the optical absorbance was 0.10 greater than that of the blank wells (which do not contain primary antibody). For the analysis of guinea pig antisera, purified recombinant pilin was diluted in 0.1 M Tris™ (pH 8) to a final concentration of 1 μg/mL. Aliquots (100 μL) were added to the wells of a microtiter plate (Immulon II, Nunc, Naperville IL) and incubated overnight at 4°C. The plates were washed five times with PBS containing 0.05% (v/v) Tween™-20 (PBS-T) using a Skanwash 300 plate washer (Skatron Instruments, Alexandria, VA). The wells were blocked using 200 μL

of 1% (w/v) BSA in PBS-T, washed and aliquots of antisera (diluted in 0.1% (w/v) BSA in PBS-T) were added to the wells. The plate was then washed and the bound primary antibodies were detected using 100 μL of alkaline phosphatase conjugated to rabbit anti-guinea pig IgG (heavy & light chains) (Zymed Laboratories, South San Francisco, CA) diluted 1:2000 dilution in 0.1% (w/v) BSA in 50 mM Tris™ (pH 8). The plates were washed and the color developed using 100 μL per well of p-nitrophenol phosphate (Sigma) (2 mg/mL in 0.5 M diethanolamine, 0.25 mM $MgCl_2$, pH 9.8). After 30 minutes, the reaction was stopped by adding 50 μL of 3 N NaOH. The absorbance was read in a Thermomax ELISA plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

All the animals immunized with rpilin responded very well, as demonstrated by the antigen ELISAs shown in Table 1.

Table 1

Endpoint Titers for the Binding of Pooled Guinea Pig Antisera Against Gonococcal rpilin to Purified Recombinant Gonococcal Pilin Protein*

Prep	Immunogen	Endpoint Titers		
		Week 0	Week 4	Week 6
1	r Pgh3-1 pilin (Prep 1)	≤100	51,345	494,805
2	r Pgh3-1 pilin (Prep 2)	54	30,237	594,298
3	r Pgh3-1 pilin (Prep 3)	≤100	24,830	546,682

* Three different lots of rpilin were used as immunogen. Guinea pigs were immunized (s.c.) on weeks 0

and 4 and bled on weeks 0, 4 and 6. Analyses were done on pooled sera.

Effect of Adjuvants on Immune Response

The effect of the following adjuvants upon the immune response against gonococcal rpilin was studied in mice: (a) Stimulon™ QS-21 in PBS (pH 6); (b) aluminum phosphate in PBS (pH 7); (c) MPL™ in PBS (pH 7); (d) aluminum phosphate and MPL™ in PBS (pH 7); or PBS (pH 7) only. For the analysis of the mouse antisera, the antigen ELISA protocol was modified as follows. The microtiter plates (Costar EIA/RIA, Corning Costar, Cambridge, MA) were coated with 100 μL of 1 μg/mL rpilin in PBS overnight at 37°C. The plate was washed five times using PBS containing 0.1% (v/v) Tween™-20 using a Skantron 300 plate washer. The wells were blocked with PBS containing 0.1% (w/v) gelatin and 0.02% (w/v) NaN_3. The primary antibody was diluted in PBS containing 0.1% (w/v) gelatin, 0.05% (v/v) Tween™-20 (PBS-TG) and 0.02% (w/v) NaN_3 and 100 μL aliquots were incubated in the microtiter plate for 2 hours. After washing, the bound primary antibody was detected using biotinylated rabbit anti-mouse IgG (Fc region) (Brookwood Biomedical, Birmingham, AL) diluted 1:8000 in PBS-TG and 0.02% (w/v) NaN_3. The plate was washed and the secondary antibody was detected, in turn, using streptavidin conjugated horseradish peroxidase diluted 1:5000 (Zymed Laboratories) in PBS-TG and 0.02% (w/v) NaN_3 (30 minute incubation). The plate was washed and the color was developed using 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M citrate (pH 4.2) containing 0.03% (v/v) hydrogen peroxide for 30 minutes and monitored at 405 nm using an SLT 340 ATTC microplate reader (SLT Labinstruments, Research Triangle Park, NC). The data

were plotted using a log-log plot and the endpoint titers were determined as previously described.

When the rpilin was adjuvanted with MPL™, a humoral immune response in mice was obtained, which was similar in magnitude to that seen with Stimulon™ QS-21 (Table 2A). Analysis of vaginal washes from the same animals also revealed a demonstrable IgG titer in the vaginal washes of these animals (Table 2B).

Table 2A

Effect of adjuvants on the immune response to gonococcal rpilin*

Humoral:

		Whole Cell ELISA				
	Antigen ELISA	Homologous Strain	Heterologous Strains			
Adjuvant	rpilin	Pgh3-1	LB2	#11	T-1	I756
$AlPO_4$	361,260	249,410	93,444	141,622	42,535	89,615
MPL™	1,262,735	902,891	499,223	586,642	165,301	321,340
$AlPO_4$+ MPL™	1,159,670	265,269	112,856	160,814	41,368	110,162
Stimulon™ QS-21	4,915,200	1,053,446	478,338	440,014	111,753	333,341

Table 2B

Effect of adjuvants on the immune response to gonococcal rpilin*

Vaginal Lavages**:

Adjuvant	rpilin Titers		Whole Cell Titers***	
	IgG	IgA	LB2 P+	LB2 P-
$AlPO_4$	1,452	262	68	≤50
MPL™	6,409	26	1,128	0
$AlPO_4$ + MPL™	938	12	120	≤50
Stimulon™ QS-21	2,716	175	311	37

* Balb/c mice (5 per group) were immunized with 10 μg of rpilin adjuvanted as described above on week 0, 4 and 6. The animals were bled on weeks 0, 4, 6 and 8. Analyses were done on pooled sera from week 8. Endpoint titers for week 0 were ≤ 5,000.

** Pooled vaginal lavages were obtained on week 8.

*** Whole cell ELISA using dried cells.

P+: piliated cells. P-: non-piliated variants derived from piliated parental cells.

Example 14
Gonococcal Whole Cell ELISA

The expression by the rpilin of a significant number of the cross-reactive epitopes found on the intact, assembled pili was subsequently verified in the whole cell ELISA, where antisera against purified rpilin exhibited high titers against numerous gonococcal strains expressing heterologous pili. The ability of guinea pig antisera to bind to live gonococcal cells was done using the following protocol. Microtiter plates (Immunolon II) were incubated overnight with 100 μL of 0.01% (w/v) poly-L-lysine (Sigma) in PBS at 4°C. Overnight agar cultures of bacteria were harvested into PBS with a Dacron swab and the turbidity of the suspension adjusted to an A_{600} = 1.0. The poly-lysine solution was discarded from the microtiter plate and 100 μL aliquots of the bacterial suspension added to the wells. The plates were washed five times with PBS using a hand-held Nunc plate washer and blocked with 200 μL of PBS containing 1% (w/v) BSA (PBS-B). The plates were then washed five times with PBS and 100 μL aliquots of antisera diluted in 0.1% (w/v) BSA in PBS were added to the wells. After washing five times with PBS, the bound antisera was detected using 100 μL of the appropriate alkaline phosphatase conjugates listed above at a 1:2000 dilution in 0.1% (w/v) BSA in PBS. The plates were washed, the color developed and the absorbance read (at 30 minutes development) as described above.

When analyzed by the whole cell ELISA, the guinea pig antisera against rpilin bound to piliated isolates from diverse geographical locations, but not to the corresponding non-piliated cells of the same gonococcal strain (Table 3).

Table 3

Endpoint Titers for the Binding of Guinea Pig Antisera Against rpilin to Live Gonococcal Cells from Diverse Strains*

Strain	Source	Pgh3-1 rpilin (Prep 1)	Pgh3-1 rpilin (Prep 2)	Pgh3-1 rpilin (Prep 3)
Pgh 3-1 P+	Pittsburgh	2,940,971	2,461,272	2,373,641
Pgh 3-1 P-	Pittsburgh	<250	<250	<250
LB2 P+	Unknown	505,439	481,815	825,517
LB2 P-	Unknown	187	34	114
I-756 P+	Ontario, Canada	689,309	713,904	1,026,231
1948 P+	Romania	290,367	259,487	325,640
1635P P+	Panama	2,714,006	≥546,750	≥546,750
22714FB P+	Fort Bragg, NC	397,763	346,895	498,882
T-1 P+	Unknown	189,490	317,370	272,828
M-2 P+	Unknown	124,906	117,734	151,966
3138K P+	Korea	220,730	179,127	584,044

Table 3 (continued)

Endpoint Titers for the Binding of Guinea Pig Antisera Against rpilin to Live Gonococcal Cells from Diverse Strains*

Strain	Source	Pgh3-1 rpilin (Prep 1)	Pgh3-1 rpilin (Prep 2)	Pgh3-1 rpilin (Prep 3)
N-2 P+	Norfolk, VA	350,210	337,232	435,164
J474B P+	Jamaica	238,640	205,247	380,970
Fresh Clincal Isolates				
52407 P+	Tampa, FL	598,124	890,678	778,607
#11 P+	Rochester, NY	214,673	292,358	385,985
FA1090:				
2-57-U17 P+	Chapel Hill, NC	1,364,126	2,379,267	3,791,263
2-42-U14 P+	Chapel Hill, NC	1,449,757	1,964,641	11,619,267

* Three different lots of rpilin were used as immunogen. Guinea pigs were immunized (s.c.) and bled as previously described. Pooled sera from week 6 bleed were assayed. Endpoint titers for week 0 sera were ≤250.

P+: piliated cells. P-: non-piliated variants derived from piliated parental cells.

The whole cell ELISA analysis of mouse antisera against rpilin (Table 2) was done using microtiter plates in which the bacteria were dried down in the wells by the following protocol. Overnight agar cultures of bacteria were harvested into PBS with a Dacron swab and the turbidity of the suspension adjusted to an A_{600} = 0.1. Aliquots (100 μL) of the bacterial suspension were added to the wells and the plate was air dried at 37°C. After evaporation of all the liquid, the plates were sealed and stored at 4°C until used. The remainder of the assay was done following the protocol previously described for the antigen ELISA of mouse antisera. The data from the whole cell ELISAs (Tables 2 and 3) suggest that rpilin induces antibodies which bind to conserved epitopes on the surface of piliated gonococci.

Example 15

Induction of a Mucosal Immune Response Against Gonococcal rpilin

Because gonorrhea is a disease of the genital mucosae, it was of interest to examine the ability of mucosal immunization to induce a mucosal immune response. This was accomplished in the following manner. Mice were immunized intranasally with gonococcal rpilin in saline (1 or 10 μg in 10 μL) with or without 1 μg of native cholera toxin on weeks 0, 1 and 2. Groups of five Swiss-Webster mice were immunized intranasally with rpilin with or without cholera toxin on weeks 0, 1 and 2. Analyses were conducted on pooled sera. Endpoints titers for week 0 were <50.

As shown in the following Table 4, there was a significant immune response detected in the antigen ELISA when the animals were immunized with rpilin in the absence of adjuvant (the week 0 titers were <300). This response was enhanced by the addition of native cholera toxin.

Table 4

Endpoint Titers for the Binding of Pooled Mouse Antisera Against Gonococcal rpilin to Purified Recombinant Gonococcal Pilin Protein

Antigen (dose μg)	Adjuvant (dose μg)	Day 22	Day 36	Day 50
rpilin (1)	none	1,684	1,287	2,291
rpilin (10)	none	29,046	20,658	71,067
rpilin (1)	cholera toxin (1)	4,673	7,526	3,273
rpilin (10)	cholera toxin (1)	107,011	321,714	280,079
None	cholera toxin (1)	<300	<300	<300

Next, these sera were examined for their ability to bind to intact, piliated gonococcal cells by an ELISA performed against cells from *N. gonorrhoeae* strain FA1090. The cells were dried down onto the microtiter plate as described previously. As shown in Table 5, a low titer was detected for rpilin alone (the week 0 titers were <300). The binding to piliated cells was greatly enhanced by addition of cholera toxin.

Table 5
Endpoint Titers for the Binding of Pooled Mouse Antisera Against Gonococcal rpilin to Whole, Piliated Gonococcal cells

Antigen (dose μg)	Adjuvant (dose μg)	Day 22	Day 36	Day 50
rpilin (1)	none	1,009	852	729
rpilin (10)	none	6,009	6,252	5,564
rpilin (1)	cholera toxin (1)	1,133	1,456	1,048
rpilin (10)	cholera toxin (1)	209,522	57,767	38,127
None	cholera toxin (1)	239	<500	<500

Example 16
Gonococcal Immunoelectron Microscopy

Visualization of the binding of antisera to piliated bacteria was conducted using the following protocol. Gold coated grids were spotted with an aliquot from a late log phase liquid culture of recA⁻, piliated *N. gonorrhoeae* for five minutes and the excess fluid was removed with a piece of filter paper. The grids were blocked with PBS-B for five minutes, followed by 1% (w/v) fish gelatin (Fluka, Ronkonkoma, NY) in PBS for 10 minutes. The grids were then incubated with polyclonal antisera diluted in PBS-B for 1-60 minutes at room temperature. Unbound antibodies were removed by floating the grid on droplets of PBS-B (4 x 30 seconds). The bound primary antibodies were detected by floating the grid on a drop of 12 nm colloidal gold bound to donkey anti-guinea pig IgG (Jackson Research Labs, West Grove, PA) diluted 1:5 in PBS-B for 30 minutes. The grids were then washed five

times on droplets of PBS-B as described above. The sample was then stabilized with 1% (v/v) glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for three minutes, then rinsed 5 x 1 minute in distilled water and lightly stained using NanoVan stain (NanoProbes, Stony Brook, NY) (pH 8) for 30 seconds. All liquid was removed by touching the grid to a piece of filter paper and examined on a Zeiss 10C transmission electron microscope at 15-75,000X using an acceleration voltage of 80 kv.

As shown by immunoelectron microscopy (Figure 1A), antibodies against the recombinant pilin were bound along the length of the heterologous pili filaments on the surface of gonococci. This suggested that the antibodies would bind to these epitopes which would be present on the surface of the bacteria *in vivo*.

Example 17

Whole Cell ELISA of Gonococcal rpilin Oligomer

In order to distinguish the biochemical and immunological properties of rpilin from those of intact pili (or pilin oligomer), purified rpilin was converted to rpilin oligomer by dialysis against pH 12 phosphate buffer. Antisera induced by this material (rpilin oligomer) was examined for its ability to bind to live, piliated gonococci using the whole cell ELISA. As shown in Table 6, antisera against rpilin oligomer had significantly lower endpoint titers for binding to diverse piliated gonococcal cells as compared to antisera induced by untreated rpilin. This suggests that rpilin oligomer had lost a significant number of the cross-reactive epitopes normally present on the rpilin protein.

Table 6

Effect of pH 12 ("oligomerization") on the Endpoint Titers for rpilin Guinea Pig Antisera Binding to Piliated *N. gonorrhoeae* Cells*

Strain	rpilin	rpilin Oligomer**
I-756	927,564	16,903
FA-19	107,100	1,982
FA1090 (2-57-U17)	721,786	6,737
LB2	905,711	4,205
#11	225,602	6,999
#4	288,120	8,104
3138K	106,315	5,429
T-1	497,987	42,162
1948	166,864	8,616
J474B	576,640	25,002

* Guinea pigs (4 per group) were immunized (s.c.) with 20 μg of rpilin antigen adjuvanted with 25 μg of Stimulon™ QS-21 on weeks 0 and 4. Pooled sera from week 6 were analyzed.

**Purified rpilin was dialyzed against 37 mM sodium phosphate (pH 12) for 48 hours at 4°C, followed by dialysis against PBS for 24 hours.

Example 18

Inhibition of Adherence to Human Cervical Cells

Because pili mediate the initial binding of *N. gonorrhoeae* to human mucosal cells, the ability of rpilin antibodies to inhibit the attachment of these

bacteria to epithelial cells was investigated. ME-180 cells, which were derived from a cervical carcinoma, were selected. In addition, to minimize the clumping of the piliated bacteria in these experiments, they were grown in liquid suspension cultures. This required using recA$^-$ derivatives of gonococcal strains Pgh3-1 or I756 in order to maintain pili expression. These recA$^-$ strains did not show significant clumping during the 4-5 hours growth in liquid culture, which made it easier to interpret the results.

In these experiments, an eight well chamber slide (Nunc) was seeded with ME-180 cells such that the cells were 80-90% confluent on the day of the experiment. An overnight culture of recA$^-$ gonococcal cells was swabbed into PBS (warmed to 37°C) and used to inoculate a flask of liquid GC media supplemented with 0.4% (w/v) $NaHCO_3$ to a final A_{600} = 0.2. The cell culture was incubated with shaking at 37°C at 120 RPM for approximately four hours, at which point the culture reached an $A_{600} \cong 0.8$. The bacterial cell suspension was diluted 1:8 in RPMI 1640. The wells of a second 8 well chamber were incubated for at least one hour with 300 μL of RPMI 1640 and fetal calf serum. The RPMI 1640 block was discarded and 40 μL of antisera or RPMI 1640 (without calf serum) added, followed by 260 μL of the diluted bacterial suspension ($\approx 8 \times 10^7$ CFU) and incubated for one hour at 37°C/5% (v/v)CO_2. The chamber well slide with the ME180 cells was washed once with RPMI 1640 devoid of antibiotics. Then, pre-incubated mixtures of bacteria and antisera were added onto the ME180 cells and the slides incubated for 30 minutes at 37°C. The media containing unbound bacterial cells was removed from the cervical cell monolayer and the wells washed gently three times with RPMI 1640. After the last wash, the chamber wells were

removed from the slide, the cells fixed in methanol for 30-60 seconds and stained with Wright-Giemsa stain (VWR Scientific, West Chester, PA). After destaining in water, coverslips were mounted over each well.

The slides were examined by light microscopy using oil immersion and pictures of representative views were taken by a person blinded as to the test sera. The resulting pictures were analyzed by persons who were also blinded as to the identity of the samples. In addition, because the piliated gonococci bound the epithelial monolayers in clumps, the effect of antisera on the binding of the gonococcal cells was quantitated by counting clumps of bacteria instead of individual bacteria. The numbers of clumps of piliated bacteria observed in ten random scans across each well were determined. The percent difference between wells containing immune and normal sera was determined. Again, this analysis was done independently by researchers blinded as to the samples that they were analyzing.

Initial results demonstrated that piliated and non-piliated cells had different binding patterns to the confluent monolayers of ME180 cells. Both piliated strains of gonococci typically bound as bacterial clumps to selected epithelial cells within the confluent monolayer. In contrast, the corresponding isogenic non-piliated bacteria either did not bind (Pgh3-1) or showed a low level, disperse binding (I756) to the epithelial monolayer. Thus, the aggregation of the bacteria on the epithelial monolayer correlated with the presence of pili.

Next, the ability of guinea pig antisera against Pgh3-1 rpilin to inhibit the binding of piliated cells of strain I-756 to ME-180 cells was tested. Analysis of representative pictures (compare

Figure 2A (week 6) to Figure 2B (week 0)) demonstrated that antibodies against rpilin significantly inhibited the binding of piliated gonococci to cervical epithelial cells. In contrast, the rpilin antisera had no effect on the binding of non-piliated cells from the same strain when compared to normal guinea pig sera (data not shown). While the number of bacteria bound under these conditions could not be determined, adherence was quantitated by counting the bacterial clumps bound in the presence of either normal or immune sera. Using this method, it was determined that antisera against rpilin resulted in ≈60% decrease in the bacterial clumps bound to epithelial cells as compared with normal guinea pig serum.

While this assay did not yield readily quantifiable data, the estimates obtained by counting bacterial clumps probably resulted in an underestimation of the effectiveness of the antisera. This is because of the binding mediated by other cell surface components (e.g., Opa proteins) which would not be expected to be overcome with antisera against rpilin alone.

Example 19

Analytic Methods for Meningococcal Chimeric Class I rpilin

The analytic methods described in Example 10 were used for the chimeric meningococcal class I rpilin. As determined by MALDI-TOF mass spectroscopy, the subunit molecular weight of the meningococcal chimeric class I rpilin protein is 17,659 daltons, which is very similar to the anticipated mass of 17,676 daltons based on the amino acid content. When this protein was analyzed by size exclusion chromatography

using a Superose™ 12 column equilibrated in PBS containing 0.05% (w/v) Zwittergent™ 3-14, the chimeric class I rpilin has an apparent molecular weight of 69,480 daltons. This is essentially identical to the apparent molecular weight of gonococcal rpilin (68,899 daltons) analyzed under the same conditions.

The N-terminal sequence of the purified meningococcal class I rpilin protein was determined by Edman degradation and the results (from three different samples) agreed with the predicted protein sequence. At least 35 residues were determined for all sequences.

Example 20
Meningococcal Chimeric Class I rpilin ELISA

The endpoint titers against purified proteins or bacterial cells were determined by ELISA using the methods described in Examples 13 and 14. The ELISAs were performed using pooled sera from the respective bleeds. Whole cell ELISA was done on meningococcal cells which had been heat killed (56°C for 60 minutes) or dried down directly onto the microtiter plates. The cell suspension was diluted to an absorbance of 0.1 at 620 nm and 100 μL aliquots were placed into the wells of microtiter plates. Each plate was dried at 37°C or at room temperature, sealed and stored at 4°C until used. The protocol for the whole cell ELISA was modified as follows: (1) primary and secondary antisera were diluted in PBS containing 0.1% (v/v) Tween™-20 and 0.1% (w/v) BSA; and (2) the plates were washed five times with PBS containing 0.05% (v/v) Tween™-20 using a Skanwash 300 plate washer.

All the guinea pigs immunized with the chimeric class I rpilin responded very well, as demonstrated by the antigen ELISAs shown in Table 7.

Table 7

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Purified Meningococcal Chimeric Class I rpilin*

	Adjuvant:		
Bleed:	Stimulon™ QS-21	$AlPO_4$	None
Week 0	23	12	32
Week 4	26,607	12,067	4,829
Week 6	1,519,956	372,539	302,911

* Guinea pigs (four per group) were immunized (s.c.) on weeks 0 and 4 with 20 μg of chimeric class I rpilin adjuvanted with (a) 25 μg of Stimulon™ QS-21 in PBS (pH6); (b) 100 μg of $AlPO_4$ in saline; or (c) PBS (pH7) only. The animals were bled on weeks 0, 4 and 6. Pooled sera was used for all analyses.

Significant responses were also seen with the ELISAs to piliated cells, as shown in Table 8.

Table 8

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Piliated Cells from *N. meningitidis* (strain H355)*

	Adjuvant:		
Bleed:	Stimulon™ QS-21	$AlPO_4$	None
Week 0	28	19	53
Week 4	1,293	1,052	381
Week 6	61,497	25,477	16,467

* Cells were heat killed.

Effects of Adjuvants on Immune Response

The effect of adjuvants upon the immune response against the meningococcal chimeric class I rpilin was studied in mice using the methods described in Example 13. As shown in Table 9, the most significant response for the binding of antisera with the meningococcal chimeric class I rpilin was achieved with the addition of Stimulon™ QS-21.

Table 9

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Purified Meningococcal Chimeric Class I rpilin*

	Adjuvant:				
Bleed:	Stimulon™ QS-21	$AlPO_4$	MPL™	$AlPO_4$/MPL™	None
Week 0	<50	44	<50	31	<50
Week 4	44,011	29,925	40,146	110,093	<250
Week 6	707,084	103,437	284,455	137,686	115,022

* Mice (ten per group) were immunized (s.c.) on weeks 0 and 4 with 10 μg of meningococcal chimeric class I rpilin adjuvanted with (a) 25 μg of Stimulon™ QS-21 in PBS (pH 6); (b) 100 μg of $AlPO_4$ in saline; (c) 50 μg MPL™ in PBS (pH 7); (d) 100 μg of $AlPO_4$ and 50 μg MPL™ in saline; or (e) PBS (pH 7) only. The animals were bled on weeks 0, 4 and 6. Pooled sera was used for all analyses.

As shown in Table 10, the most significant response for the binding of antisera to piliated cells from *N. meningitidis* was also achieved with the addition of Stimulon™ QS-21.

Table 10

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Piliated Cells from *N. meningitidis* (strain H355)*

	Adjuvant				
Bleed:	Stimulon™ QS-21	$AlPO_4$	MPL™	$AlPO_4$/MPL™	None
Week 0	172	146	153	160	<50
Week 4	6,088	2,310	4,205	5,647	311
Week 6	171,718	25,135	52,053	17,039	16,617

* Cells were heat killed.

Further analyses demonstrated that this antisera against the chimeric class I rpilin bound to meningococcal cells which expressed either class I pilin or, in some cases, class II pilin. The results, shown in Table 11, evidence this partial cross-reactivity.

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Piliated Cells from *N. meningitidis**

Strain	Class Pilin expressed	Adjuvant: Day 0	Stimulon™ QS-21	$AlPO_4$	MPL™
H355	I	409	127,383	41,190	102,987
M982	I	217	>36,540	>36,540	>36,540
CDC1521	II	988	2,602	1,345	1,768
FAM18	II	3,518	>36,540	26,513	>36,540

* Cells were dried down (at room temperature) directly onto microtiter plates without being heat killed.

Example 21

Meningococcal Chimeric Class I rpilin Immunoelectron Microscopy

Visualization of the binding of chimeric meningococcal class I rpilin antisera to piliated cells from *N. meningitidis* strain H355 was conducted using transmission-electron microscopy as follows. A colony from an overnight culture of *N. meningitidis* was carefully picked up using a sterile loop and placed in a microfuge tube containing 0.5-1.0 mL of modified Franz media [1.3 g/L glutamic acid, 20 mg/L cysteine, 10 g/L $Na_2HPO_4 \cdot 7H_2O$, 90 mg/L KCl, 6 g/L NaCl, 2 g/L yeast dialysate and supplemented with dextrose (4 g/L), glutamic acid (100 mg/L), cocarboxylase (200 μg/L) and ferric nitrate (5 mg/L)]. Gold coated grids were spotted with an aliquot of the cell suspension for five

spotted with an aliquot of the cell suspension for five minutes and the excess fluid was removed with a piece of filter paper. The bacterial cells were then fixed with 4% (v/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde in PBS for 30 minutes at room temperature. The grids were incubated, in sequence, with (a) PBS-B for five minutes, (b) 1% (w/v) fish gelatin in PBS for 10 minutes, and (c) PBS containing 0.2 M glycine for five minutes. The blocked grids were then probed with antisera against meningococcal chimeric class I rpilin protein as described in Example 16. As shown in Figure 3A), the antibodies against the meningococcal chimeric class I rpilin protein bound along the length of the pili. In contrast, normal serum (week 0) did not show any binding to the pili (Figure 3B).

Example 22

Cross-reactivity of Meningococcal Chimeric Class I rpilin Antisera with Gonococcal Piliated Cells

Based on the sequence similarity of meningococcal class I pilin and gonococcal pilin, it was shown in Example 4 above that antisera directed against gonococcal rpilin recognized and bound to piliated meningococcal cells. In this example, it is demonstrated that antisera raised against meningococcal chimeric class I rpilin binds to piliated gonococcal cells. The data from the mouse and guinea pig experiments are summarized in Tables 12 and 13, respectively.

Table 12
Endpoint Titers for the Binding of Mouse Antisera Against Meningococcal Chimeric Class I rpilin to Piliated *N. gonorrhoeae* Cells

Antigen / Adjuvant

GC Strain	Nm rclass I pilin					GC rpilin + MPL™
	Stimulon™ QS-21	$AlPO_4$	MPL™	$AlPO_4$/ MPL™	None	
I756	4,527,943	79,927	114,958	56,627	57,426	356,936
FA1090	531,627	40,406	97,224	31,267	38,122	219,600

Table 13
Endpoint Titers for the Binding of Guinea Pig Antisera Against Meningococcal Chimeric Class I rpilin to Piliated *N. gonorrhoeae* Cells

Antigen / Adjuvant

GC Strain	Nm rclass I pilin			GC rpilin + Stimulon™ QS-21
	Stimulon™ QS-21	$AlPO_4$	None	
I756	301,969	122,714	78,111	46,424
FA1090	322,311	262,422	170,842	108,094

GC - *N. gonorrrhoeae*; Nm - *N. meningitidis*

Example 23

Passive Protection Against Meningococcal Bacteremia by Meningococcal Chimeric Class I rpilin Antisera

An accepted animal model for evaluating the ability of vaccines to protect against meningococcal bacteremia is the infant rat model originally described by Saukkonen and Leinonen (34). The ability of guinea pig antisera against meningococcal chimeric class I rpilin (adjuvanted with Stimulon™ QS-21) to protect against bacteremia caused by a meningococcal strain, which expresses a pilin, was tested. On day 0, Sprague-Dawley infant rats (4-5 days old) were passively immunized (i.p.) with 0.1 mL of guinea pig antiserum (week 6) against chimeric class I rpilin diluted 1:5, 1:10 or 1:20 in PBS. The control group received 0.1 mL injection of normal guinea pig serum (week 0) diluted 1:5 in PBS. Twenty-four hours later, the animals were challenged i.p. with approximately 5 x 10^5 colony forming units (cfu) in 0.1 mL of piliated *N. meningitidis* (strain H355). Three hours after challenge, the animals were sacrificed and aliquots of cardiac blood were diluted and plated onto GC agar plates. The plates were then incubated for 18-24 hours at 37°C with 5% CO_2. The bacterial colonies were counted and the level of bacteremia was then determined. One way analysis of variance t test was used to compare groups receiving immune serum (week 6) with the control group receiving normal serum (week 0). As shown in Table 14 below, animals passively immunized with guinea pig antiserum specific for meningococcal chimeric class I rpilin showed more than a log reduction in the level of bacteremia as compared to those animals immunized with normal guinea pig serum.

This difference was statistically significant, with a p value of <0.05.

Table 14

Ability of Guinea Pig Antisera Against Meningococcal Chimeric Class I rpilin Protein to Prevent Bacteremia in Infant Rats Challenged with Piliated *N. meningitidis* (Strain H355)*

Bleed	Dilution	Mean cfu ± std
Week 0	1:5	4.87 ± 0.18
Week 6	1:5	3.55 ± 0.48**
Week 6	1:10	3.63 ± 0.36**
Week 6	1:20	3.98 ± 0.75**

* Antisera against meningococcal chimeric class I rpilin protein was obtained from guinea pigs as described in Table 7.

** p <0.05.

cfu ± std = colony forming units ± standard deviation.

Example 24

Induction of a Mucosal Immune Response Against Meningococcal Chimeric Class I rpilin

Mice were immunized intranasally with meningococcal chimeric class I rpilin in saline (5 μg in 10 μL) with or without 1 μg of a cross-reactive mutant form of cholera toxin (CT-CRM, E29H) on weeks 0, 1 and 2. As shown in the following Table 15, there was a significant immune response detected in the antigen ELISA when the animals were immunized with rpilin in the absence of adjuvant. This response was enhanced by the addition of cholera toxin.

Table 15

Endpoint Titers for the Binding of Pooled Mouse Antisera Against Meningococcal Chimeric Class I rpilin to Purified Recombinant Meningococcal Chimeric Class I rpilin Protein*

	rpilin, no adjuvant	rpilin plus CT-CRM
Sera		
IgG	6,168	1,181,871
IgA	490	3,940
Bronchial wash**		
IgG	<10	580
IgA	<10	19
Nasal wash**		
IgG	<10	98
IgA	12	236
Vaginal wash**		
IgG	174	70
IgA	15	687

* Pooled sera from week 4 were analyzed. Endpoint titers for pooled sera from week 0 for IgG and IgA were <50.

** Washes were performed as follows:

Bronchial: Lungs were washed 5 times with 1 mL RPMI 1640, then 50 µL fetal bovine serum (FBS) was then added to the sample, which was clarified by

centrifugation (12,000 x g x 5 minutes) and stored at -20°C.

Nasal: The nasal passages were washed once with 0.5 mL of RPMI 1640 and 20 µL of FBS was then added to the sample before storage at -20°C.

Vaginal: Vaginas were washed 5 times with 0.075 mL of RPMI 1640 and 10 µL of FBS was then added to the sample before storage at -20°C.

Example 25

Active Protection Against Meningococcal Colonization by Meningococcal Chimeric Class I rpilin Antisera

The initial step in meningococcal disease in human beings is the colonization of the nasopharynx by the bacteria. In this process, pili are believed to play a major role in mediating the inital attachement to the epithelial cells. A number of researchers have described procedures for colonizing the nasophayrnx in neonatal animals, but no one has investigated this as a model for testing the efficacy of meningococcal vaccines (35). In order to assess the invention described herein, a nasal colonization model for *N. meningitidis* using adult outbred mice has been developed. Swiss-Webster mice were immunized with meningococcal chimeric class I rpilin adjuvanted with MPL™ subcutaneously on weeks 0, 4 and 8. At week 10, the animals received an intraperitonal injection of 2 mg iron dextran (Sigma) and were challenged intranasally with approximately 1 x 10^7 cfu of mid-log phase piliated meningococci in a volume of 10 µL which also contained 40 µg of iron dextran. On day 1 after challenge, half the animals received an additional intraperitonal injection of 2 mg iron dextran. The

number of viable bacteria in the nose were determined on days 1 and 2 after challenge by plating nasal tissue homogenates on GC agar plates containing selective antibiotics. The results are shown in Table 16.

Table 16

Number of Viable Bacteria (cfu) Recovered from Nasal Homogenates of Mice Challenged with Piliated *N. meningitidis* Strain H355*

Antigen (Dose µg)*	cfu per nose	
	Day 1**	Day 2**
H355 Whole cell (25)	1,165	67
Class I rpilin (10)	6,866	63
Saline	17,943	3,406

* All vaccines were formulated with 100 µg of MPL™ per dose. Each group consisted of five mice.
** Days after intranasal challenge.

Example 26

Western Blot Analysis of the Immune Response Against Meningococcal Class II Chimeric rpilin

The purified meningococcal class II chimeric rpilin was used to immunize guinea pigs following the protocol described in Example 11. The antisera derived from guinea pigs immunized with meningococcal class II chimeric rpilin were analyzed first by Western blots (data not shown). These blots demonstrated that the antisera against meningococcal class II chimeric rpilin recognized a band corresponding to pilin in whole cell lysate from piliated meningococcal cells which expressed either class II pilin (FAM18) or class I pilin (H355). In contrast, antisera directed against

an extract from *E. coli* containing the pTrcHis vector only did not react with either pilin band in these Western blots.

Example 27

Binding of Antisera against Meningococcal Chimeric Class II rpilin to Piliated Meningococcal Cells

Antisera elicted against partially purified meningococcal chimeric class II rpilin was shown to bind to meningococcal cells from the homologous strain, FAM18 with a titer of >36,450 (the week 0 titer was 473).

Bibliography

1. Tennent, J.M., and Mattick, J.S., pages 127-146 of Fimbriae. Adhesion, genetics, biogenesis and vaccines, P. Klemm, Ed. (CRC Press, Boca Raton, FL 1994).

2. Strom, M.S., and Lory, S., Ann. Rev. Microbiol., 47, 565-596 (1993).

3. Heckels, J.E., Clin. Microbiol. Rev., 2 (Suppl.), S66-73 (1989).

4. U.S. Patent Number 4,702,911.

5. Schoolnik, G.K., J. Exp. Med., 159, 1351-1370 (1984).

6. Davies, J.K., et al., pages 147-155 of Fimbriae. Adhesion, genetics, biogenesis and vaccines, P. Klemm, Ed. (CRC Press, Boca Raton, FL 1994).

7. Aho, E.L., et al., Infect. Immun., 65, 2613-2620 (1997).

8. Achtman, M., et al., J. Inf. Dis., 165, 53-68 (1992).

9. Levine, M.M., et al., pages 255-270 of Fimbriae. Adhesion, genetics, biogenesis and vaccines, P. Klemm, Ed. (CRC Press, Boca Raton, FL 1994).

10. Stewart, D.J., et al., Aust. Vet. J., 62, 153-159 (1985).

11. Tramont, E.C., Clin. Microbiol. Rev., 2 (Suppl.), S74-7 (1989).

12. Boslego, J.W., et al., Vaccine, 9, 154-162 (1991).

13. Rothbard, J.B., and Schoolnik, G.K., Adv. Exp. Med. Biol., 185, 247-73 (1985).

14. Olafson, R.W., et al., Infect. Immun., 48, 336-342 (1985).

15. Seifert, H.S., et al., Vaccine, 6, 107-109 (1988).

16. Sparling, P.F., et al., Proc. Natl. Acad. Sci. USA, 91, 2456-2463 (1994).

17. Rothbard, J.B., et al., Proc. Natl. Acad. Sci. USA, 82, 915-919 (1985).

18. Forest, K.T., and Tainer, J.A., pages 167-173 of Vaccines 97. Molecular Approaches to the Control of Infectious Diseases, F. Brown, et al., Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1997).

19. Robinson, E.N.J., et al., Mol. Microbiol., 3, 57-64 (1989).

20. Eisentein, B.I., et al., pages 89-113 of Recombinant DNA vaccines: rationale and stratagies, R.E. Isaacson, Ed., (Marcel Dekker, Inc., New York, NY 1992).

21. Lepper, A.W., Aust. Vet. J., 65, 310-316 (1988).

22. Hull, R.A., et al., Infec. Immun., 33, 933-938 (1981).

23. Granted European Patent Number 202,260 B1.

24. Hoyne, P.A., et al., J. Bact., 174, 7321-7327 (1992).

25. Elleman, T.C., et al., Infect. Immun., 51, 187-192 (1986).

26. O'Meara, T.J., et al., Immun. Cell. Biol., 71, 473-488 (1993).

27. Emery, D.L., et al., Aust. Vet. J., 61, 237-8 (1984).

28. Stewart, D.J., et al., Vet. Microbiol., 27, 283-93 (1991).

29. Alves, A.M.B., et al., American Society for Microbiology, Abstract E-79, page 253 (1997).

30. Meyer, T.F., et al., Cell, 30, 45-52 (1982).

31. Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

32. Stephens, D.S., Clin. Microbiol. Rev., 2 (Suppl.), S104-111 (1989).

33. Tramont, E.C., et al., J. Clin. Invest., 68, 881-888 (1981).

34. Saukkonen, K. and Leinonen, M., pages 815-820 of Gonococci and Meningococci, J. T. Poolman, Ed. (Kluwer Academic Publications, Dordrecht, Netherlands, 1986).

35. Salit, I.E., et al., Can. J. Microbiol., 30, 1022-1029 (1984).